REMARKS

Claims 1-15 presently appear in this case. No claims have been allowed. The official action of October 25, 2006, has now been carefully studied. Reconsideration and allowance are hereby respectfully urged.

Briefly, the present invention relates to the use of certain synthetic peptides for the treatment of systemic lupus erythematosus (SLE). The synthetic peptides are based on a complementarity-determining region (CDR) of the heavy or light chain of a pathogenic anti-DNA monoclonal antibody that induces a SLE-like disease in mice, or a salt or derivative thereof, as well as dual synthetic peptides and peptide polymers based thereon.

It has been noticed that the substitute specification submitted on October 1, 2004, inadvertently failed to include the amendments to the specification made in the Preliminary Amendment filed July 17, 2003. Accordingly, the present amendment amends the substitute specification to put it in the form of the originally filed specification as amended on July 17, 2003.

Claims 1-15 have been rejected under 35 U.S.C. 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had

possession of the claimed invention. The examiner considers that there is insufficient written description with respect to the peptide "reaction product thereof with an organic derivatizing agent capable of reacting with selected side chains or terminal residues, which reaction product retains at least a portion of the function of the peptide to inhibit specifically the proliferative response in cytokine secretion of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies." The examiner states that there is sufficient reason to doubt that any peptides, let alone derivatives, meeting the limitations of the claims exist. The examiner states that given the disclosure of no examples of the derivatives employed in the claimed method, one of skill in the art would conclude that the specification fails to disclose a representative number of species to describe the claimed genus. This rejection is respectfully traversed.

As will be discussed below with respect to the enablement rejection, there is sufficient evidence to establish that peptides of the present invention function as disclosed. As to the derivative language, this is the exact same language that this same examiner approved during the prosecution of 08/913,994, the parent application of the present application, which issued as patent no. 6,613,536. The law has not changed between 2003 and 2007, nor has the examiner, nor has the

specification. Accordingly, if the derivative language was acceptable in 2003, it should still be acceptable now.

As to the examiner's statement that the claims encompass the use of a genus of derivatives that might include no peptides or that might be essentially unlimited, this statement is not understood. The claim language is based on the definition of the term "chemical derivative" appearing in paragraph [0035] of the present specification. The definition does not involve changing amino acids, but merely derivatizing. All of the chemical derivatives have the specified amino acid formulas and therefore it is clear that the inventors were in possession of such derivatives at the time the invention was made. Claim 1 does not read on all analogs that retain at least a portion of the function of the peptide, but only on reaction products of the peptide having the specified sequence with an organic derivatizing agent that reacts only with selected side chains or the terminal residue of the peptide and, thus, does not change its basic sequence. Such derivatization is commonly done in the preparation of pharmaceuticals from peptides and it is believed inequitable for the examiner to require applicants to dedicate to the public such minor derivatization of the peptides of the present invention, particularly if those peptides are considered otherwise to be allowable. The examiner has not objected to the "salts" as also claimed. What is the

difference between an acid addition salt of a peptide and another organic derivatization of a side chain or terminal residue of a peptide? Just as salts have been considered to be acceptable, so should reaction products as now claimed in claim 1.

It is assumed that this rejection has been made as it is tied in with the enablement rejection and if the enablement rejection can be overcome, then the written description rejection will be overcome. Accordingly, for the reasons discussed below with respect to the enablement rejection as well as to reasons presented herein, reconsideration and withdrawal of this rejection are respectfully urged.

Claims 1-15 have been rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The examiner states that the specification provides insufficient evidence that the claimed method would effectively treat SLE. The examiner states that while the mechanism of action for the method of the instant claims is not disclosed, it appears to require inducing tolerance to self peptides as part of a treatment for an autoimmune disease. The examiner cites anecdotal references to cases where success in animal models designed for tolerance induction did not work with human clinical trials, and the examiner cites one instance where a clinical trial was suspended due to adverse reactions. Thus,

the examiner concludes that in view of the quantity of experimentation necessary, the lack of sufficient guidance in the specification, the lack of sufficient working examples, the unpredictability of the art, and the breadth of the claims, it would take undue trial and error to practice the claimed invention. This rejection is respectfully traversed.

First, the examiner is incorrect in stating that altered peptide ligands (APLs) of self peptides are employed in the present invention. The present invention is not an APL, it is a region of a CDR of an antibody. Thus, one cannot predict that the present invention will fail in clinical trials for the same reason as the failed mechanisms of the references cited by the examiner.

The examiner states that a review of the instant specification shows no induction of tolerance and that the examples involve only the use of three CDR-derived peptides in *in vitro* proliferation assays. The examiner states that the inventors apparently conclude that tolerance was induced given reduced proliferation, but that this minimal evidence comprises an insufficient showing that the claimed method can effectively treat SLE without stimulating an immune response.

To the contrary, however, the present specification has examples that teach more than what the examiner speaks of. Example 7 shows that mice are tolerized by means of the present

invention. This example was published in the Waisman et al. paper, *PNAS* 94:4620-4625 (1997), a copy of which is attached hereto. Newborn mice were tolerized with peptide Ia or a control peptide. Later, the mice were immunized with mAb 5G12, which is known to induce SLE in mice upon immunization. Later, the sera of the mice were tested for the presence of anti-DNA or anti-NE antibodies, which are indicative of an immune response to the immunization by the monoclonal antibody 5G12. It was shown that the autoantibody titers in the sera of the experimental mice that were tolerized with peptide Ia were not significant with respect to either DNA or nuclear extract antigens, whereas the mice tolerized to control peptide prior to their immunization with this antibody, produced high autoantibody titers. Thus, this experiment is more than just a proliferation test, but is an actual antibody test that indicates that neonatal tolerization with the peptide Ia could lower levels of autoantibodies in the sera of mice later immunized to produce SLE.

Examples 8 and 9 are proliferation tests. In Example 8, the mice were immunized with peptides Ia and IIIa in CFA. In Example 9, they were immunized with mAb 5G12 in CFA. In both of the examples, those mice were treated with the same peptides in PBS. This effectively is a tolerizing administration. When lymph node cells were later taken from these mice, the ones that

were not treated with the peptide in PBS proliferated in the presence of the antigen. However, the lymph node cells from the mice that had been treated showed that such proliferative responses were greatly inhibited. This confirms, in T-cells, tolerization shown with antibodies in Example 7.

In Example 10, the specification shows that these results are relevant to the human antibody. In Example 10, the mice were immunized with human mAb 16/6 Id in CFA and then were treated with either peptide Ia or IIIa in PBS. The same type of proliferation of lymph node cells was found in the control mice as was found in Examples 8 and 9, and the same type of inhibition of such proliferation was found in the treated mice. This shows that the peptides of the present invention were also effective in tolerizing against the human antigen.

This data in the specification does indeed show that, whatever the mechanism, there are results that are consistent with the induction of tolerance. The results are more than *in vitro* proliferation assays as they involve actual *in vivo* tolerization of mice; only the test results are conducted *in vitro*. It also involves more than reduced proliferation, which is significant, but it also reduced antibody production. This is more than minimal evidence and is a sufficient showing to establish that applicant is not merely proposing an unproved hypothesis or engaging in a "respectable guess." Indeed, such

experimentation was considered significant enough to warrant publication in *PNAS*, which is a major journal.

Subsequent experimentation has confirmed the results that are in the specification. Thus, for example, Eilat et al., *J. Clin. Immunol.*, 20:268-278 (2000), copy attached, shows that in a mouse model for spontaneous development of SLE, the development is inhibited by tolerization with the peptides in accordance with the present invention. The Zinger et al. paper, *International Immunology*, 15:205-214 (2003), copy attached, uses the same mouse model of spontaneous SLE but treats the mice after development of symptoms, after the disease is already established. The ameliorating effect of the CDR based peptides of the present invention was shown. In Fig. 2 it was actually shown to ameliorate the kidney disease caused by SLE, which is a very significant result. In Eilat et al., *PNAS*, 98:1148-1153 (2001), copy attached, the results of treatment by means of the present invention of mice whose SLE was induced by the human 16/6 Id antibody, show that such SLE could be treated using the peptides of the present invention. Brosh et al., *Autoimmunity*, 211-219 (2002), copy attached, establishes that administration of pCDR3 to mice immunomodulated an established experimental SLE. In Sthoeger et al., *Clin. Exp. Immunol.* 131:385-392 (2003), copy attached, pCDR1 and pCDR3 of both murine and human anti-DNA antibodies were shown to immunomodulate SLE-associated

responses of PBL (peripheral blood lymphocytes) of SLE human subjects. The Summary on page 385 concludes:

> Over all, the results of our study demonstrate that the CDR-based peptides are capable of down-regulating *in vitro* autoreactive T cell responses of PBL of SLE patients.

The examiner has cited *Rasmusson v. SmithKlein Beecham Corp.*, 75 USPQ2d 1297, 1302 (Fed. Cir. 2005) for the proposition that enablement cannot be established unless one skilled in the art would accept without question an applicant's statements regarding an invention, particularly in the absence of evidence regarding the effect of a claimed invention. Without proof, mere plausibility would be the test and applicants could obtain rights to inventions consisting of little more than respectable guesses as to the likelihood of their success. However, as discussed above, the present specification is not simply a "respectable guess." Applicant's statements regarding the invention are supported by substantial proof using several different mechanisms and several different CDRs. Furthermore, the substantial literature from the laboratory of the present inventors in highly respected journals shows that the statements made in the present specification are not just lucky guesses but are the result of substantial academic research and experimentation.

With respect to the examiner's reference to problems noted in clinical trials relating to APLs, those of ordinary

skill in the art understand that the failure of a clinical trial does not necessarily mean that experimental results cannot be expected to be transferred to humans. For example, attached hereto is a press release from the National Institutes of Neurological Disorder and Stroke, entitled, "MS Clinical Trials Confirm Approach, Demonstrate Need to Refine Targeted Peptide Therapy." This relates to the clinical trial of the approach using APLs for treatment of MS. While the clinical trials were suspended, NIH says that the adverse reactions highlight the importance of proceeding carefully with clinical research, even when an experimental therapy looks promising, and that more carefully controlled clinical trials are necessary. Thus, the reports cited by the examiner do not necessarily lead a person of ordinary skill in the art to the conclusion that clinical trials will never work in a certain category of treatment, although one can conclude that more carefully controlled trials are necessary. Accordingly, we cannot agree with the examiner that the results in some other clinical trials using other types of active agents require the conclusion that one can never trust experimental results in small animals. One can never get a patent if one must wait for clinical results before filing the application. Reference is made to MPEP 2107.03(IV) "Human Clinical Data" where it states:

> Office personnel should not impose on applicants the unnecessary burden of providing

> evidence from human clinical trials. **There is no decisional law that requires an applicant to provide data from human clinical trials to establish utility for an invention related to treatment of human disorders** (see *In re Isaacs*, 347 F.2d 889, 146 USPQ 193 (CCPA 1963); *In re Langer*, 503 F.2d 1380, 183 USPQ 288 (CCPA 1974)), even with respect to situations where no art-recognized animal models existed for the human disease encompassed by the claims. *Ex parte Balzarini*, 21 USPQ2d 1892 (Bd. Pat. App. & Inter. 1991) (human clinical data is not required to demonstrate the utility of the claimed invention, even though those skilled in the art might not accept other evidence to establish the efficacy of the claimed therapeutic compositions and the operativeness of the claimed methods of treating humans). Before a drug can enter human clinical trials, the sponsor, often the applicant, must provide a convincing rationale to those especially skilled in the art (e.g., the Food and Drug Administration) that the investigation may be successful. Such a rationale would provide a basis for the sponsor's expectation that the investigation may be successful. In order to determine a protocol for phase I testing, the first phase of clinical investigation, some credible rationale of how the drug might be effective or could be effective would be necessary. Thus, as a general rule, if an applicant has initiated human clinical trials for a therapeutic product or process, Office personnel should presume that the applicant has established that the subject matter of that trial is reasonably predictive of having the asserted therapeutic utility. [Bold emphasis added; underlined emphasis original]

While the specific CDRs used in the present claims have not yet commenced human clinical trials. The data in the specification and in the publications attached hereto would normally be sufficient to permit the FDA to grant an IND for phase I

testing. Thus, the examiner should accept that this data is reasonably predictive of having the asserted therapeutic utility. For all of these reasons, reconsideration and withdrawal of the enablement rejection are respectfully urged.

It is submitted that all of the claims now present in the case clearly define over the references of record and fully comply with 35 U.S.C. 112. Reconsideration and allowance are therefore earnestly solicited.

Respectfully submitted,

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National Institute of Neurological Disorders and Stroke

MS Clinical Trials Confirm Approach, Demonstrate Need to Refine Targeted Peptide Therapy

For release: Sunday, October 01, 2000

Two clinical trials of a targeted peptide therapy in patients with relapsing-remitting multiple sclerosis (MS) have been halted due to adverse effects in some study participants. Despite these adverse effects, the findings confirm that the targeted peptide plays a role in the disease and provide valuable information that may help refine this type of therapy for MS as well as other autoimmune diseases. Results of the two studies will be published in the October 1, 2000, issue of *Nature Medicine*. (1)(2)

Investigators at the National Institutes of Health, Stanford University, and 13 other research centers tested a type of targeted immunotherapy designed to alter immune reactions to a specific protein, thereby treating the disease without compromising other immune functions. In this case, the therapy was an altered version of one segment of a protein called myelin basic protein (MBP). Therapies using altered peptides of this type are referred to as altered peptide ligand or APL therapies. MBP is a component of myelin, a fatty substance that surrounds and insulates nerve fibers. It is released during flare-ups of relapsing-remitting MS, and it is believed to be one of the proteins which is attacked by the immune system, causing symptoms of the disease.

Relapsing-remitting MS is marked by periodic increases in brain inflammation and disease symptoms, interspersed with periods of full or partial recovery. Previous research suggested that introducing an altered form of MBP into MS patients might cause the body to react to the normal MBP in a protective way instead of a harmful way, thereby stopping flare-ups of the disease.

In the first study, researchers led by Roland Martin, M.D., of the National Institute of Neurological Disorders and Stroke (NINDS) Cellular Immunology Section tested the therapy in 24 patients with active relapsing-remitting MS. The study was partially sponsored by Neurocrine Biosciences, Inc., which owns the rights to this therapy, under a Cooperative Research and Development Agreement (CRADA). After studying the patients' normal disease patterns for 6 months, the researchers administered 50 mg of APL weekly for up to 9 months while using clinical tests, immunology work-ups, and MRI scans to carefully monitor the effects of the therapy in patients. Two out of eight treated patients showed an increase in inflammatory brain lesions that was linked to the APL therapy by sophisticated studies of their immune cells. One patient developed a general hypersensitivity reaction and three others discontinued dosing due to side effects that could not be directly linked to the therapy. Hypersensitivity is a condition in which the body over-reacts to a protein or other stimulus. After the first seven patients showed adverse effects, the dose was reduced to 5 mg for the eighth patient. When this patient also showed an increase in MS lesions that appeared to be related to the therapy, the trial was halted prematurely.

In the second study, Ludwig Kappos, M.D., of the University of Basel, Switzerland, Lawrence Steinman, M.D., of Stanford University Medical School, and collaborators at 14 research centers in the United States, Europe, and Canada conducted a randomized, double-blind clinical trial designed to test the therapy in 144 patients. The study was sponsored by Neurocrine Biosciences, Inc., and by Novartis. After a 1-month monitoring period, patients in this trial received either a placebo or a 5-, 20-, or 50-mg dose of the therapy weekly for 4 months. Patients were then offered the therapy at a dose of 5 mg weekly. In this study, the researchers found no significant difference in the frequency or number of relapses in patients receiving the therapy vs. the placebo, although the volume of new inflammatory brain lesions was reduced in some patients who received the 5-mg dose of the therapy. However, 9 percent of the patients enrolled in the study developed systemic hypersensitivity to the therapy. These reactions caused the trial to be stopped after only 53 patients had completed the double-blind phase. The scientists point out that neither of the studies tested the therapy in enough patients to show whether it can actually relieve patients' symptoms.

While the adverse effects in these studies were disappointing, the increase in brain lesions in some patients proves that MPB can induce an immune response in MS and is therefore a good target for MS immunotherapy, the researchers say. "There is no longer any doubt in my mind that MBP is one target autoantigen (a protein that triggers an immune response) in MS," says Dr. Martin.

These studies provide valuable information about how to design, refine, and test targeted immunotherapies. Results from both studies suggest that lower doses of this therapy are better than higher doses. The adverse reactions found in these studies were not predicted by previous studies in animals or humans, which highlights the importance of proceeding carefully with clinical research even when an experimental therapy looks promising, says Dr. Martin. The NIH researchers also feel their study demonstrates the importance of immunological tests to determine how targeted immunotherapies work in patients.

While these studies provide valuable information about APL therapies, researchers need to determine why some individuals responded differently than others to this therapy and what dose, frequency, and mode of administration provide the best results. More carefully controlled clinical trials of this or similar therapies should eventually reveal the answers to these questions.

The NINDS, part of the National Institutes of Health in Bethesda, Maryland, is the nation's leading supporter of research on the brain and nervous system. The NINDS is now celebrating its 50th anniversary.

(1) Bielekova B., Goodwin B., et al. "Encephalitogenic potential of myelin basic protein peptide (83-99) in multiple

sclerosis — results of a phase II clinical trial with an altered peptide ligand." *Nature Medicine*, October 2000, Vol. 6, No. 10, pp. 1167-1175.

(2) Kappos L., Comi G., et al. "Induction of a non-encephalitogenic Th2 autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo controlled, randomized phase II trial. " *Nature Medicine,* October 2000, Vol. 6, No. 10, pp. 1176-1182.

Originally prepared by Natalie Frazin, NINDS Office of Communications and Public Liaison.

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Related Items
Fact Sheet

Autoimmunity, 2002 VOL. 35 (3), pp. 211-219

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Treatment of Induced Murine SLE with a Peptide Based on the CDR3 of an Anti-DNA Antibody Reverses the Pattern of Pathogenic Cytokines

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A peptide based on the complementarity detennining region (CDR) 3 of a pathogenic anti-DNA monoclonal antibody that bears the 16/6 idiotype (Id) was shown previously to be a dominant T-cell epitope in experimental SLE, and to be capable of inhibiting SLE-associated responses. When injected, concomitant with active immunization with the pathogenic human anti-DNA, 16/6 Id+ mAb, pCDR3 inhibited the proliferation of LN-derived T cells stimulated *in vitro* with the 16/6 Id mAb. The inhibition of the specific proliferative responses could be reversed by the addition of exogenous IL-2 to the cultures. Analysis of secreted cytokine profile in supernatants of these cultures demonstrated that pCDR3 treatment reduced significantly the levels of both IL-2 and IPN-I' that were elevated further in cells of the 16/6 Id-immunized mice. The CDR3-based peptide was shown here to immunomodulate *in vivo* experimental SLE, induced by the human anti-DNA 16/6 Id+ antibody. The beneficial effects of pCDR3 on the clinical manifestations of SLE were associated with downregulation of the Th1-type (IL-2, IPN-I') and proinflammatory (TNF-cx) cytokines, whereas the immunosuppressive cytokine TGF-13 was up regulated.

Keywords: Experimental lupus; CDR-based peptide; Immunomodulation of cytokines; Down-regulation of SLE

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by the production of autoantibodies to nuclear proteins and nucleic acids, and involving multiple organ systems and diverse clinical manifestations.[1,2]

Induction of experimental SLE in mice was reported previously by our laboratory, using the human,i31 or murine [4] monoclonal anti-DNA antibodies that bear the common idiotype (Id) 16/6. The 16/6 Id-immunized mice produced high levels of autoantibodies, including anti-DNA and anti-nuclear protein antibodies, as well as anti-idiotypic antibodies of the 16/6 Id network. Additionally, SLE afflicted mice developed leukopenia, proteinuria and high-intensity immune complex deposits in their kidneys.[3,4] Induced experimental SLE was found to share features with the (NZBxNZW)F1 spontaneous SLE model. Thus, sequencing of the variable regions coding for the heavy and light chains of anti-DNA mAb isolated from mice afflicted with experimental SLE, show high homology with the variable regions of anti-DNA mAb isolated from (NZBxNZW)F1 mice.[5]

Two peptides based on the sequence of the complementarity determining regions (CDR) of the pathogenic murine monoclonal anti-DNA, 16/6 Id+ antibody (designated 5G12)[4] were synthesized. The CDR3-based peptide was shown to induce T-cell proliferation and mild SLE upon active immunization of SJL mice. [6] Furthermore, when administered in PBS, the CDR3-based peptide was able to inhibit T-cell stimulation in mice that are actively immunized with the same peptide, or with the whole 16/6 Id-bearing pathogenic antibodies of either murine or human origin. [6]The CDR3-based peptide was shown to down regulate 16/6 Id-induced lupus.[7] The involvement of pCDR3 in the pathogenicity of murine SLE was further supported by showing that syngeneic immunization with a T-cell line specific to pCDR3 induced mild experimental disease in SJL mice.[Sl Also, naive splenocytes of (NZBXNZW)Fl lupus prone mice were shown to proliferate in response to pCDR3. [9]

Cytokines, as mediators of immune responses, play a role in the pathogenesis of autoimmunity. Many abnormalities of the cytokine network have been described in patients with SLE and in murine models of lUpUS.[IO,II]In contrast to organ-specific diseases, the

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Thlrrh2 paradigm does not apply to lupus, as both Thl and Th2 cytokines have been found to exert profound and complex effects on mouse models of this disease. [12] We have shown previously that induction of experimental SLE in mice was accompanied by elevated production of Th 1-type (IL-2 and IFN--y) cytokines, while disease progresslOn was charactenzed by an Increase In the Th2-type (IL-4 and IL-lO) cytokines and a decrease in the Th-l type cytokines. Full-blown disease was characterized by diminished levels of IL-2, IFN--y and IL-4, while levels of IL-lO remained elevated. Secretion of the proinflammatory cytokines IL-l and TNF-a were high throughout the experimental period. [13] Studies analyzing the effects of administration of recombinant cytokines and monoclonal antibodies and the use of gene knock-out and transgemc mice have produced data that support a role for both Thl and Th2 subsets in lupus.[14]

In the present report, we have investigated the mechanisms by which pCDR3 exerts its immunomodulatory effects. Injection of pCDR3 concomitant with active immunization with the 16/6 Id mAb inhibited the *in vitro* recall response to the antibody, and reduced 16/6 Id-specific secreted levels of the Thl-type cytokines, IL-2 and IFN--y. Additlon of exogenous IL-2 to the cultures reversed the inhibited state by resuming proliferation to the anti-DNA mAb. Treatment of animals, inflicted with experimental SLE, at the time of disease induction, modulated the pathogenic cytokine pattern, stimulated specifically by the 16/6 Id mAb. Thus, pCDR3 restored the Thl-type cytokine (IL-2, IFN--y) and TNF-a profile to levels similar to those observed in normal mice, while enhancing the immunosuppressive cytokine TGF-I3. These changes were associated with improvement of the clinical manifestations and significant amelioration of the typical kidney damage.

MATERIALS AND METHODS

Animals

SJL inbred female mice (Jackson Laboratory, Bar Harbor, USA) were used at the age of 8-10 weeks.

Synthetic Peptides

Peptides pCDR3 (Y-Y-C-A-R-F-L- W-E-P- Y-A-M-D- Y-W-G-Q-G-S), based on the CDR3 of the murine anti-DNA monoclonal antibody 5G12,[4] and reversed pCDR3 (S-G-Q-G-W-Y-D-M-A-Y-P-E-W-L-F-R-A-C-Y-Y), were prepared using an automated multiple peptide synthesizer (model AMS 422; Abimed Langenfeld, Germany) using the company's protocols for t-butyloxycarbonyl (t-BOC) strategy. [15]

A fb d' n I o les

The human 16/6 Id anti-DNA mAb (IgGl/k) bearing the 16/6 Id was previously described.[16] The antibody was secreted by hybridoma cells grown in culture and was purified on a protein G-Sepharose column (Pharmacia, Sweden). Human IgG (ChromoPure, whole molecule, Jackson Immunoresearch) was used as control.

Immumza .on and Induct Ion of Experlmen taI SLE

Mice were injected intradermally into the hind footpads with 2 f.Lgofthe 16/6 Id mAb, or human IgG, emulsified in complete Freund's adjuvant (CFA). For experimental SLE induction, mice were immunized with the 16/6 Id, and boosted three weeks later with the 16/6 Id (2 f.Lg)in PBS in a total volume of 100 f.Ll.

Treatmen t of Experlmen taI SLE Wlh pCDR3

Mice immunized with the 16/6 Id were injected concomitant with 250 f.Lg/animal of pCDR3 in PBS subcutaneously, or with the vehicle (PBS) only. The animals were similarly treated with the pCDR3 once a week for additional three weeks.

Prolhera tlve Responses of Lymp h Node-derlve d T C ll c s

Eleven days after immunization with the 16/6 Id (with or without treatment with pCDR3), lymph nodes (LN) were harvested and pooled. Cells (10^6/well) were cultured in the presence of 0.5-50 f.Lg/mlof the 16/6 Id. Cultures were established in enriched medium containing 1% syngeneic normal mouse serum in flat-bottom microtiter plates (200 f.LIIwell). At the end of a 4-day incubation period, 0.5 f.LCi of eH}-thymidine (25 Ci/mmol, Amersham Pharmacia Biotech) was added to the cultures. Sixteen hours later, cells were harvested and radioactivity measured by a 13counter

Regeneration of 16/6 Id-specific Proliferative Response in T Cells Derived from pCDR3-treated Mice

Eleven days after immunization with the 16/6 Id_6 mAb, lymph nodes were harvested and pooled. Cells (10 /well) were cultured in the presence of 25 f.Lg/mlof the 16/6 Id with, or without, exogenous recombinant mouse IL-2 (Pharmingen) at 250, 500 or 1000 units/ml. Cultures were established as described above. At the end of a 4-day incubation period, 0.5 f.LCiof eH}-thymidine was added to the cultures. Sixteen hours later, cells were harvested and radioactivity measured.

DNA-specific Antibody Assay

Detection of specific antibodies in sera of immunized mice was carried out by ELISA. Briefly, flat-bottom maxi-sorb plates (Nunc, Roskilde, Denmark) were coated with 5 f.Lg/ml polY-L-lysine (Sigma), and then coated with 5 f.Lg/mllambda phage dsDNA (Worthington biochemical corporation, NJ), overnight. The plates were then washed

and blocked with 10% FCS. Diluted sera were then added, followed by incubation with peroxidase-conjugated goat anti-mouse IgG (gamma chain Fc specific, Jackson Immuno Research, PA). The reaction was developed with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, Si ma).

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Detetion f Clinical and Pathological SLE-associated Mamfestatlons

Proteinuria was measured by a semi-quantitative method, using a Combistix kit (Ames Division, Bayer Diagnostics, Slough, UK). Levels of proteinuria were calculated according to the manufacturer's scale where: + 1 equals 0.3 g/l, +2 equals 1 g/l, +3 equals 3 g/l. For immunohistology, frozen kidney cryostat sections (6 fLm) were fixed and stained with FITC-conjugated goat antibodies to mouse IgG, I'-chain specific (Jackson Immunoresearch, West Groove, PA, USA). Significance was tested using the Mann - Whitney test.

Induc tIon of Cytok IDe Pro duc t.on

Mice immunized with the human 16/6 Id mAb and either treated or not with pCDR3 were sacrificed at different periods. Spleen and lymph nodes were harvested and cells were incubated (5×10^6/ml for splenocytes and 4×10^6/ml for lymph node cells) in the presence of the 16/6 Id mAb. Supernatants were collected 24, 48 or 72 h after the establishment of the culture and kept m - 80°C.

Analysis of Cytoplasmic Cytokines

Mice immunized with the human 16/6 Id mAb and either treated or not with pCDR3 were sacrificed at different periods after the treatment. Lymph nodes were harvested and cells (1 X 106) were treated with a Cytoperm kit (Serotec) according to the manufacturer's protocol. Thereafter, the cells were incubated with different FITC-conjugated anti-cytokine antibodies or with FITC-conjugated isotype-matched controls. Cells were assessed by the FACSort cytometer and the data was analyzed using the CELLQUEST software.

Detect Ion of Cytok IDes ID Superna tan ts

Measurements ofIL-2, IFN-I' and TNF-a were performed by ELISA using antibodies to the various cytokines (OPTEIA kits, PharMingen) according to the manufacturer's protocol. For the detection of acid-activated TGF-13,plates were coated with recombinant human TGF-131 sRII/Fc chimera (R&D System) and the second antibody used was biotinylated anti-human TGF-131 (R&D System). The substrate solution used was TMB color reagent (Helix Diagnostics).

RESULTS

Effects of the CDR3-based Peptide on 16/6 Id-stimulated T-cell Responses

In order to elucidate the mechanism(s) by which pCDR3 down regulates experimental SLE induced by the human 16/6 Id mAb, we first analyzed the effects of this peptide on short-term 16/6 Id mAb-stimulated T-cell responses. As can be seen in Fig. 1, pCDR3 injected *in vivo,* concomitant with active immunization by the 16/6 Id mAb, inhibited (up to 60% in this specific experiment) the proliferation of LN-derived T cells, stimulated *in vitro* with the 16/6 Id mAb (Fig. 1A). The inhibition of 16/6 Id-stimulated proliferation, conferred already *in vivo* by pCDR3, could be reversed by the addition of exogenous IL-2 to the 16/6 Id-stimulated cultures (Fig. IB). Analysis of secreted cytokines in supernatants collected from the cultures showed that IL-2 and IPN-I' were induced by stimulation with the 16/6 Id mAb, and that the *in vivo* treatment with pCDR3 reduced significantly the levels of the latter Thl-type cytokines (Fig. 1C). These results are representative of three similar experiments, consisting of 10-12 animals each.

Effects of the CDR3-based Peptide on 16/6 Id-induced Experimental SLE

In VIeW of the results described above, we attempted the analyses of the means by which pCDR3 exerted its immunomodulatory effects, *in vivo,* on experimental SLE induced by immunization with the human anti-DNA 16/6 Id. Thus, pCDR3 was administered (subcutaneously, 250 fLg/mouse) once a week for 4 weeks, at the time of disease induction. The animals were examined every 4 weeks for serum autoantibodies and urinary protein. The results shown in Table I are representatives of two long-term experiments involving 64 and 90 animals, respectively. As can be seen in the Table, anti-dsDNA antibody levels measured 4 months after disease induction were only slightly reduced by the treatment with pCDR3. Levels of proteinuria were lower in the pCDR3-treated mice as compared to untreated, 16/6 Id mAb immunized animals (Table I). Immunohistological examination of kidney sections was carried out at the end of the experiment. The results summarized in Table I demonstrate that, as compared to untreated animals, pCDR3 treatment reduced significantly the percentage of animals with immune complex deposits (from 87.5 to 37.5%) and also the mean density of the already formed lesions (from 2.75 ± 0.7 to 0.5 ± 0.75). It is noteworthy that treatment with pCDR3 inhibited even the background of immune complex deposits typical to normal aged SJL mice (Table I and Fig. 2D). Figure 2 represents immunohistology of kidney sections' of mice

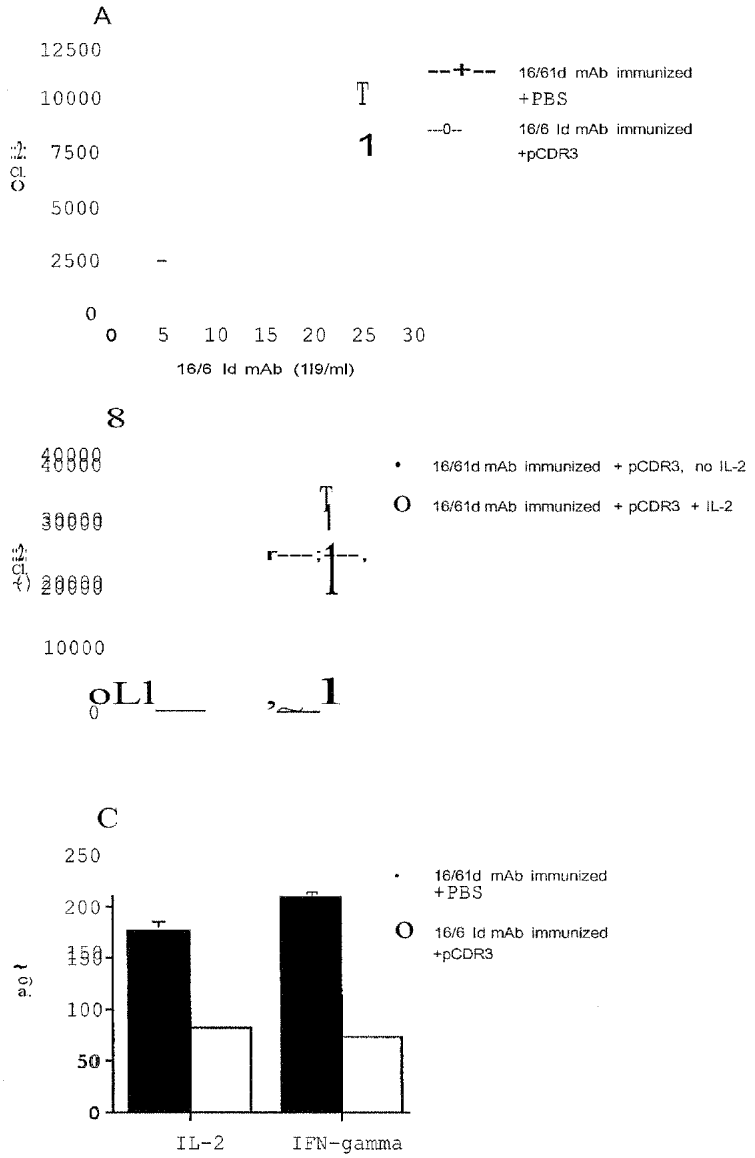

FIGURE 1 Inhibitory effects on the proliferation of LN-derived T cells from mice immunized with the 16/6 Id and treated with pCDR3. (A) SJL mice were immunized with 2 flog/animal of the 16/6 Id mAb in CFA and concomitantly injected with 250 flogof pCDR3 i.p. in PBS (o), or with PBS, as control (8). For the induction of the recall response *in vitro,* popliteal LN were harvested 11 days post immunization and cells (106/well) were incubated in triplicate wells with different concentrations of the 16/6 Id (0.5-25 flog/ml). Results of the proliferative responses to the 16/6 Id mAb are expressed as CPM ± SD of triplicate wells. CPM values of cells with medium were 825 ± 269. CPM values for cells with Con A were 14526 ± 1299. (B) Exogenous recombinant mouse IL-2 (2000 units/ml) was added (D), or not (.), to culture wells containing LN cells derived from animals immunized with 16/6 Id mAb and concomitantly treated with pCDR3. Cells were stimulated *in vitro* with the 16/6 Id mAb (25 flog/ml). Results are expressed as CPM ± SD of triplicate wells. (C) Secreted cytokines were measured in supernatants collected from cultures derived from mice immunized with the 16/6 Id mAb and treated with PBS (.), or from mice immunized with the 16/6 Id mAb and treated with pCDR3 (D). The cells were stimulated *in vitro* with the 16/6 Id mAb (25 flog/ml) and the specific cytokines were measured as described in "Materials and Methods" section. Results are expressed as pg/ml ± SD. Results of all three parts (A, B, C) are representative of three similar experiments.

immunized with the 16/6 Id mAb and either not treated (Fig. 2A), treated with the vehicle PBS (Fig. 2B), or with pCDR3 (Fig. 2C). Kidney sections of normal age-matched SJL mice are presented in Fig. 2D. The figure demonstrates the significant reduction of immune complex deposits in kidneys of the pCDR3-treated mice.

Analysis of the Cytokine Profile in Animals Inflicted with Experimental SLE and Treated with the CDR3-based Peptide

Every month following immunization and treatment, two animals of each group were sacrificed and examined for their cytokine profile. Cytoplasmic cytokines were

TABLE I Clinical manifestations of animals inflicted with experimental SLE and treated with the CDR3-based peptide.

Treatment	Anti-dsDNA Ab levels*	Proteinuria (g/l)†	Percentage of animals with immune complex deposits in kidneys	Immune complex deposit density in kidneys‡
16/6 Id immunized	1.63 ± 0.1	0.53 ± 0.36	87.5	2.75 ± 0.70
+pCDR3	1.36 ± 0.2	0.25 ± 0.08	37.5	0.50 ± 0.75'
+PBS	1.45 ± 0.2	0.57 ± 0.53	75.0	1.75 ± 1.28
Normal (age-matched)	0.63 ± 0.1	0.21 ± 0.08	61.5	1.08 ± 1.12

* Serum anti-dsONA Ab levels were tested and are presented as mean values (at a dilution of 1: 10) of absorbance at 415 nm ± SO.
† Mean values for urinary protein (± SO) were derived based on the numeric values specified by the manufacturer (see "Materials and methods" section).
‡ Mean immune complex density values (see "Materials and methods" section) ± SO. Results are representatives of two experiments. The data were analyzed by the Mann-Whitney test.
~ pCOR3-treated animals were compared to the 16/6 Id mAb immunized animals ($p = 0.0006$).

measured in a pool of LN cells of two mice that were not stimulated *in vitro* (Fig. 3A). Secreted cytokines were determined in supernatants of LN cells and splenocytes (pooled from two animals) that were stimulated *in vitro* with the 16/6 Id mAb (Fig. 3B). Regarding cytoplasmic cytokines, the most notable effect of the *in vivo* treatment with pCDR3 was observed at 60 days after the end of treatment (Fig. 3A). The LN cells of SLE-inflicted mice (16/6 Id mAb immunized) were stained positively for cytoplasmic IL-2, IL-4, IL-lO, and IFN-I'. *In vivo* treatment with pCDR3 reduced dramatically the percentage of cells with positive staining of these cytokines, bringing the values close to those measured in normal age-matched SJL LN cells (13.4% for IL-2, 19.3% for IL-4, 5.4% for IFN-I', and no detection for IL-lO).

The profile of secreted cytokines was examined in supernatants of pooled lymph node cells and splenocytes stimulated *in vitro* with the 16/6 Id mAb (Fig. 3B). The most notable effect of pCDR3 treatment was observed at day 90 after the treatment. Thus, at this time point, when clinical manifestations emerge, LN cells from animals treated with pCDR3, secreted reduced levels of both Thl-type cytokines IL-2 and IFN-I' (Fig. 3B). Results that are not shown in the figure indicated that levels of the

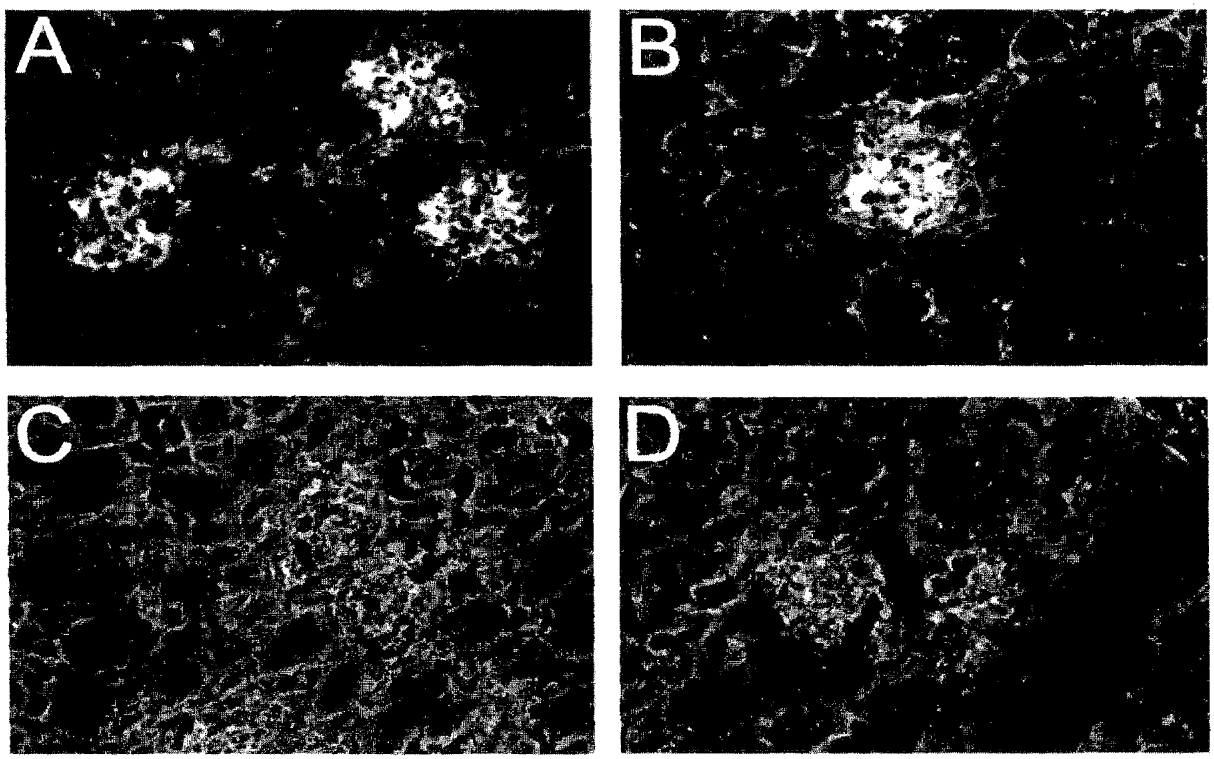

FIGURE 2 Immunohistology of kidney sections from mice immunized with the 16/6 Id and treated with pCDR3. Five months after the end of the treatment, mice were sacrificed, their kidneys removed and analyzed for the presence of immune complex deposits as described in "Materials and Methods" section. The picture represents kidney sections of mice of the different groups: 16/6 Id mAb-immunized mice (A), 16/6 Id mAb-immunized and PBS-treated mice (B), 16/6 Id mAb-immunized and pCDR3-treated mice (C), and normal age-matched SJL mice (D) (X 400). The results represent two experiments that consisted of 64 and 90 animals each.

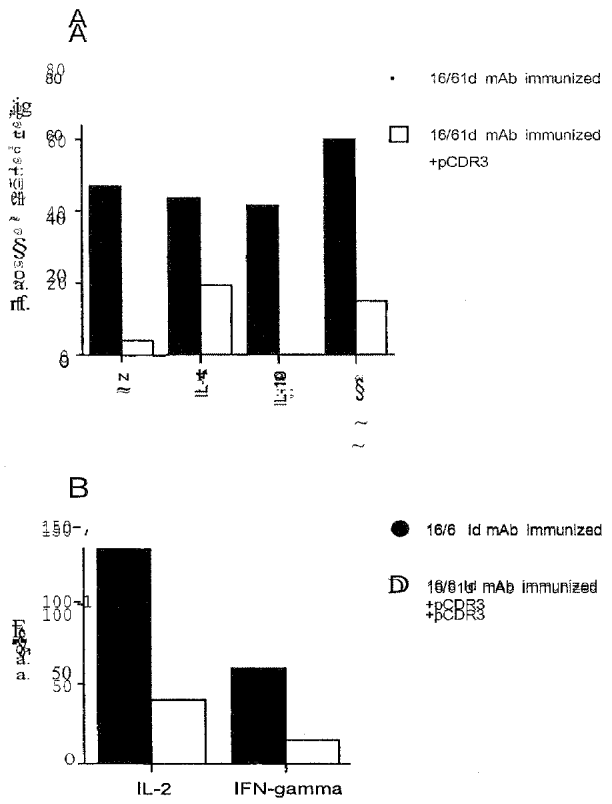

FIGURE 3 Cytoplasmic and secreted cytokines in LN cells derived from animals inflicted with experimental SLE and treated with the CDR3 -based peptide. The experiments consisted of 64 and 90 animals each. At days 30, 60, 90 and 120 following disease induction by the *16/6* Id mAb and treatment with the pCDR3, 2 animals from each group were sacrificed and their LN-derived cells were pooled. (A) Non-stimulated cells (1 X 106) from *16/6* Id mAb immunized mice (.) and from *16/6* Id mAb immunized mice treated with pCDR3 (D) were stained for the presence of intracellular IL-2, IL-4, IL-JO, and IPN-I' as described in "Materials and Methods" section. FITC-conjugated isotype matched antibodies were used as controls, The results presented in the figure were obtained 60 days after treatment and are expressed as percentage of positively stained cells out of 5000 cells analyzed. Secreted cytokines were measured in supernatants of cultured cells (5 X *J06/ml)* derived from *16/6* Id mAb immunized mice (.) and *16/6* Id mAb immunized and treated with pCDR3 (D). The cells were stimulated *in vitro* with the *16/6* Id mAb (25 *f.lg/ml)* in duplicate cultures. Secreted IL-2 (B) and IPN-I' (C) were measured by ELISA as detailed in "Materials and Methods" section. Results for secreted cytokines are shown for the time point of 90 days after treatment, secretion after 48 h. The results are expressed as pg/ml. Results are representative of two long-term experiments.

Th2-type IL-5 were elevated in LN cells derived from pCDR3-treated animals (150pg/ml) as compared to non-treated animals (low below the detection level) already 60 days after the treatment. The changes in the cytokine pattern was not observed in animals inflicted with experimental SLE and treated with reversed pCDR3 peptide, or with PBS, as controls.

Examination of the pro-inflammatory cytokine *TNF-a* was carried out both on cytoplasmic and secreted cytokine levels. As shown in Fig. 4A, the proportion of LN cells stained positively for cytoplasmic *TNF-a* increased gradually in time following disease induction by 16/6 Id mAb. The figure also demonstrates that treatment with pCDR3 changed this process by dramatically reducing the percentage of cells stained for *TNF-a,* bringing the values close to those measured in normal age-matched SJL mice. The same phenomenon was observed when we examined secreted *TNF-a* in supernatants of 16/6 Id mAb stimulated LN cells. Figure 4 represents the results determined 120 days after treatment. It is shown that animals treated with pCDR3 exhibited a dramatic reduction in *TNF-a* levels. It is noteworthy that *TNF-a* could not be detected in supernatants derived from LN of normal age-matched SJL mice. In contrast to the effects of pCDR3 on *TNF-a* levels, splenocytes of pCDR3-treated animals secreted significantly higher levels of the aCld-actlvated Immunosuppressive cytokine TGF-[3, as compared to 16/6 Id mAb immunized, non-treated mice (Fig. 4C).

DISCUSSION

The present study investigated the effects of the CDR3-based peptide on three levels, namely, *in vitro, ex vivo* and *in vivo.* The main findings of the present report were that pCDR3 exerted its beneficial effects by modulating SLE-associated T-cell responses probably in part by inducing T-cell anergy, as was demonstrated by the analysis of T-cell proliferative potential and the secreted cytokine pattern. *In vivo,* pCDR3 treatment ameliorated manifestations of experimental SLE and was associated mainly with down-regulation of the proinflammatory cytokine *TNF-a,* up-regulation of the Immunosuppressive Cytokine TGF-[3 and a decrease in the Thl-type cytokines IL-2 and IFN-'Y.

Autoreactive T cells were demonstrated in lupus.[17] Thus, investigation of lupus-associated T-cell responses may be a key for understanding the effects of pCDR3 in the 16/6 Id induced model of SLE. The CDR3-based peptide inhibited the recall T-cell response to the whole 16/6 Id mAb probably by inducing clonal anergy or tolerance. Addition of exogenous IL-2 reverse tested state of unresponsiveness of 16/6 Id-stimulated LN cells derived from pCDR3 treated animals (Fig. IB), in agreement with previous reports.[18] Clonal anergy was shown to be associated with a decrease in IL-2 and IFN-'Y production.[181] Indeed, treatment with pCDR3 concomitant willi immunization with the 16/6 Id, resulted in inhibition of the proliferative responses of LN cells to the immunizing antibody and in down regulation of IL-2 and IFN-'Y secretion (Fig. lC). Preliminary results, (Brosh, unpublished results) suggested that injection of pCDR3 concomitant with immunization with the 16/6 Id mAb reduced the proportion of the *in vitro* 16/6 Id-stimulated cells undergoing apoptotic cell death, and increased the proportion of cells accumulated in the G_0/G_1 phase of the cell cycle. Gilbert and Weigle[19] proposed that anergy was the result of G_1 cell-cycle blockade. Powell and co-authors showed that artificial (rapamycin treatment) inhibition of cell cycle progression from G_1 into the S

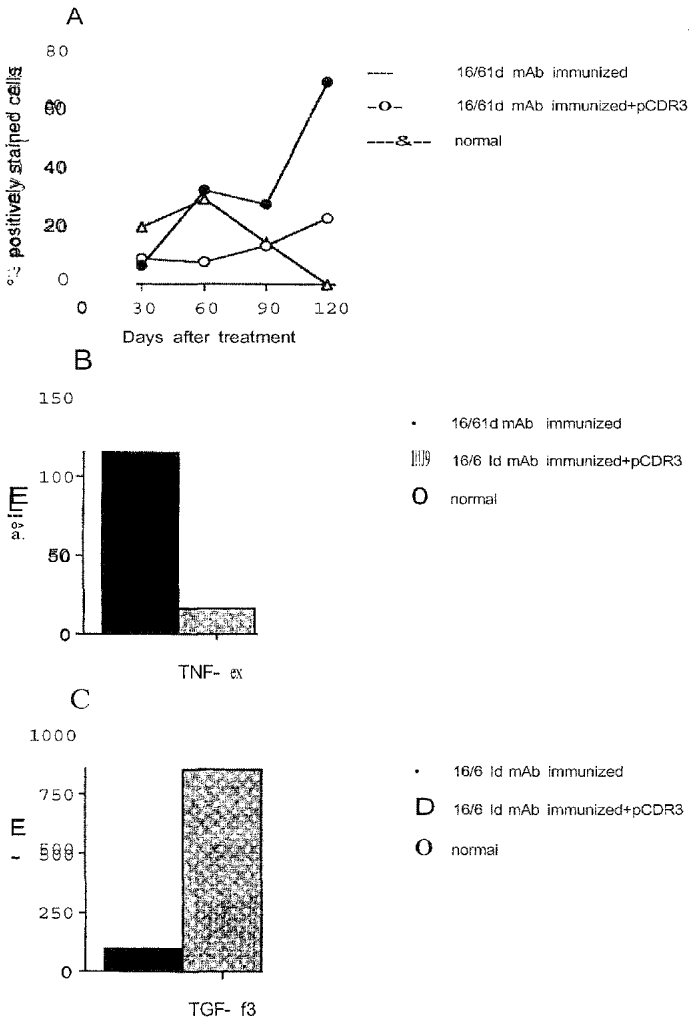

FIGURE 4 Proinflammatory versus immunosupressive cytokines in animals inflicted with experimental SLE and treated with the CDR3-based peptide. At days 30, 60, 90 and 120 following treatment with the pCDR3, 2 animals from each group were sacrificed and their LN-derived cells were pooled. (A) For the detection of cytoplasmic TNF-Ol, LN-derived cells (1 x J06) from 16/6 Id mAb immunized (8), or from 16/6 Id mAb immunized and treated with pCDR3 (O), or from normal age-matched SJL mice *(D,)*, were tested for the presence of intracellular TNF-Ol by FACS, as described in "Materials and Methods" section. FITC-conjugated isotype matched antibodies were used as controls. Results are expressed as percentage of positively stained cells. (B) For the detection of secreted TNF-Ol, LN-derived cells (5 x $J0^6$/ml) from 16/6 Id mAb immunized mice (.), or from 16/6 Id mAb immunized mice treated with pCDR3 (LilJ), or from normal age-matched SJL mice (LilJ), were stimulated *in vitro,* in duplicate cultures, with the 16/6 Id mAb *(25/LgImi)* for 48 h. The results are of cells taken at day 120 after treatment. (C) For the detection of secreted TGF-J3, splenocytes (4 X J06) derived from 16/6 Id mAb immunized mice (.), or from 16/6 Id mAb immunized mice treated with pCDR3 (LilJ), or from normal age-matched SJL mice (0), were incubated for 72 h in a serum-free medium containing 1% Nutridoma SP media supplement JOO X (Bohringer Mannheim). Secreted TNF-Ol and TGF-J3 were measured in the supernatants collected from these cultures by ELISA, as detailed in "Materials and methods" section. The results are expressed as pg/ml and are representatives of two long-term experiments.

phase rendered CD4+ Thl clone anergic. They suggested that anergy induction occurred as a result of blockade in the progression from G] to the S phase of the cell cycle. [20]

Disease activity was shown to be regulated by cytokines in patients with SLE.II6.21] Previous reports from our laboratory indicated that development of experimental SLE in mice involved two stages. Thus, disease induction was accompanied by elevated production of Thl-type (IL-2 and IFN--y) cytokines. Disease progression was characterized by a decrease in the Th-l type cytokines and an increase in the Th2-type (IL-4 and IL-lO) cytokines. At the stage of full-blown disease (approximately 7 months after induction), IL-2, IFN--y and IL-4 levels were diminished, while levels of IL-IO remained elevated. Secretion of the proinflammatory cytokines IL-I and TNF-a were shown to be high throughout the experimental periodY^3]

In this report, treatment with pCDR3 at the time of SLE induction, reduced dramatically the proportion of LN-derived cells expressing aberrant levels of cytoplasmic IL-2, IL-4, IL-lO, and IFN--y, bringing these values close to those measured in normal age-matched SJL mice. The

IL-2 expression by freshly isolated SLE peripheral blood mononuclear cells (PBMC), was found to be elevated compared to control PBMC.[22] Production of IFN-'Y from patients with SLE correlated significantly with disease activity.[23] Treatment with pCDR3 down regulated levels of secreted IL-2 and also of secreted IFN-'Y (Fig. 3). Administration of IFN-'Y along with SLE induction aggravated disease manifestations in experimental animals. [24] Further, MRL/lpr/lpr mice deficient of IFN-'Y gene, [25] or the IFN-'Y receptor gene[1261] were protected from disease development, as were (NZBxNZW)Fl mice treated with anti-IFN-'Y antibodies[27] or IFN-'Y soluble receptor. [28]

In addition to causing reduction of the Thl-type cytokines, treatment with pCDR3 reduced levels of both cytoplasmic and secreted proinflammatory TNF-a (Fig. 4). Lupus patients with active disease had significantly higher levels of serum TNF-a (and IL-6) compared to p~ len s ":1 mac .Ive Iseag u er, a me~ 0 mIce afflIcted wIth expenmental SLE wIth eIther methotrexate or tamoxifen resulted in beneficial clinical effects associated with diminished secretion of TNF-a.130.31]Moreover, mice with experimental SLE benefited sIgmficantly from treatment wIth eIther antI-TNF-a Abs or pentoxiphyline (that was shown to reduce TNF-a levels). [32]

Treatment WIth pCDR3 up regu Iated tbe secretIOn of TGF-I3. TGF-13 null mice were shown to develop autoimmune manifestations resembling SLE. [33]Ohtsuka and co-authors reported that unstImulated and antI-CD2-stimulated PBLs from SLE patients (especially the NK cell subset) produce decreased levels of active TGF-I3. [34] TGF-55]ws sbwn t0 mhb TNF-a pro ductIOn, *In vItro*. It ISnot clear yet whether m the present study, the elevated levels of TGF-13 down regulated directly TNF-a pro uc IOn.

Treatment with pCDR3 injected four times a week, ameliorated lupus-associated clinical manifestations in the 16/6 Id Immumzed mIce. The treatment reduced shghtly the levels of anti-dsDNA antibodies and proteinuria, but decreased significantly the appearance and density of Immune complex deposIts m the kidneys (Table I). The CDR3-based peptide was recently shown to prevent lupus in the (NZBXNZW)Fl mice. [36]The ability of pCDR3 to modulate lupus m (NZBXNZW)Fl mIce can be explamed based on the high sequence homology of anti-DNA antibodies isolated from lupus-prone mice[137,38]and from mIce WIth 16/6 Id-mduced lupus. Several recent papers reported the successful usage of autoantibody or autoantigen-derived pep tides in spontaneous murine lUpUSmo de ls. Thus, IIljeCtIOn o f a peptI de correspon dmg to the VH CDR3 region of a natural polyreactive autoantibody to young pre-autoimmune (NZBXNZW)Fl mIc~ dc layc dL89]evbpment o f protemuna an d mcreasc d survIval rate. In the (SWRXNZB)Fl mouse model of lupus, a therapy course with peptides derived from nuc leo some core hIstones was ellectIve m de laymg lupus nephritis in pre-nephritic mice and prolonged survival and halted progression of renal disease. [40]In another system, peptides that react with a pathogenic mouse monoclonal anti-DNA antibody protected mice from renal deposition of the antibody, *in vivo*. [41] Further, treatment with peptides derived from the VH region of anti-DNA autoantibody to young (NZBXNZW)Fl, substantially delayed development of anti-DNA antibodies and nephritis and prolonged the animals' survival. [42] In agreement, we demonstrated here that treatment of experimental SLE with a peptide based on the CDR3 of an anti-DNA autoantibody led to significant amelioration of the clinical manifestations. The latter has been associated with the immunomodulation of the pathogenic cytokine pattern.

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References

[1] Shoenfeld, Y. and Mozes, E. (1990) "Pathogenic idiotypes of autoantibodies in autoimmunity", *FASEB J.* 4,2646-2651.

[2] Kotzin, B.L. and O'Dell, J.R. (1995) "Systemic lupus erythematosus", In: Frank, M.M., Austen, K.E, Claman, H.N. and Unanue, E.R., eds, *Samter's Immunologic Diseases* (Little, Brown and Co., Boston), pp 667-697.

[3] Mendlovic, S., Brocke, S., Shoenfeld, Y., Ben Basat, M., Meshorer, A., Bakimer, R. and Mozes, E. (1988) "Induction of a systemic lupus erythematosus like disease in mice by a common human anti-DNA idiotype", *Proc. Natl Acad.* Sci. *USA* 85, 2260-2264.

[4] Waisman, A., Mendelovic, S., Ruiz, J.P., Zinger, H., Meshorer, A. and Mozes, E. (1993) "The role of the 16/6 idiotype network in the induction and manifestation of systemic lupus erythematosus", *Int. Immunol.* 5, 1293-1300.

[5] Waisman, A. and Mozes, E. (1993) "Variable regions of autoantibodies isolated from mice with experimental systemic lupus erythematosus", *Eur. J. Immunol.* 23, 1566-1573.

[6] Waisman, A., Ruiz, J.P., Israeli, E., Eilat, E., Konen-Waisman, S., Zinger, H., Dayan, M. and Mozes, E. (1997) "Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of a pathogenic anti-DNA monoclonal antibody", *Proc. Natl Acad.* Sci. *USA* 94, 4620-4625.

[7] Brosh, N., Zinger, H., Fridkin, M. and Mozes, E. (2000) "A peptide based on the sequence of the CDR3 of a murine anti-DNA mAb is a better modulator of experimental SLE than its single amino acid substituted analogs", *Cell Immunol.* 205, 52-61.

[8] Brosh, N., Eilat, E., Zinger, H. and Mozes, E. (2000) "Characterization and role in experimental SLE of T cell lines specific to peptides based on CDR1 and CDR3 of a pathogenic anti-DNA monoclonal antibody", *Immunology* 99,257-265.

[9] Brosh, N., Dayan, M., Fridkin, M. and Mozes, E. (2000) "A peptide based on the CDR3 of an anti-DNA antibody of experimental SLE origin is a dominant T cell epitope also in (NZBXNZW)F1 lupus-prone mice", *Immunol. Lett.* 72, 61-68.

[10] Handwerger, B.S., Rus, Y, da Silva, L. and Via, e.S. (1994) "The role .of cytokines in the immunopathogenesis of lupus", *Springer Semm.Immunopathol.* 16, 153-180.

[11] Horwitz, D.A. and Jacob, e.o. (1994) "Thecytokine network in the pathogenesis of systemic lupus erythematosus and possible therapeutic implications", *Springer Semin. Immunopathol. 16,* 181-200.

[12] Theofilopoulos, A.N. and Lawson, B.R. (1999) "Tumor necrosis factor and other cytokines in murine lupus", *Ann. Rheum. Dis.* 58(1), 149-155.

[13] Segal, R., Bermas, B.L., Dayan, M., Kalush, E, Shearer, G.M. and Mozes, E. (1997) "Kinetics of cytokine production in experimental systemic lupus erythematosus: involvement of T helper cell *IIT*

helper cell 2-type cytokines in disease", *J. Immunol.* 158, 3009-3016.

[14] Holdsworth, S.R., Kitching, A.R. and Tipping, P.G. (1999) "Thl and Th2 T helper cell subsets affect patterns of injury and outcome in glomerulonephritis", *Kidney Int.* 55, 1198-1216.

[15] Merrifield, R (1986) "Solid phase synthesis", *Science* 232, 341-347.

[16] Waisman, A, Shoenfeld, Y, Blank, M., Ruiz, P.J. and Mozes, E. (1995) "The pathogenic human monoclonal anti-DNA that induces experimental systemic lupus erythematosus in mice is encoded by a VH4 gene segment", *Int. Immunol.* 7, 689-696.

[17] Mao, e., Osman, G.E., Adams, S. and Datta, S.K (1994) "T cell receptor alpha-chain § repertoire of pathogenic autoantibody-inducing T cells in lupus mice", *J. Immunol.* 152, 1462-1470.

[18] Beverly, B., Kang, S.M., Lenardo, J. and Schwartz, R.H. (1992) "Reversal of *in vitro* T cell clonal anergy by IL-2 stimulation", *Int. Immunol.* 4,661-671.

[19] Gibert, KM. and Weigle, W.O. (1993) "Thl cell anergy and blockade in Gla phase of the cell cycle", *J. Immunol.* 151, 1245-1254.

[20] Powell, J.D., Lerner, C.G. and Schart~ R.H. (1999) InhIbItIon of cell cycle progressIOn by Rapamycm mduces T cell clonal anergy even in the presence of costimulation", *J. Immunol.* 162, 275-g4.

[21] ll-Pri J C lEd b D A (2000) "ean, .' ., Tyrre ce,., raw ey, . an In erg, ". ( ) Cytokines and systemIc lupus erythematosus *Ann. Rheum. DIS.* 59,243-251.

[22] HOrwltz, D.A , Wang, H. and Gray, J.D (1994) "Cytokine gene profile m ClrCUlatmg blood mononuc lear ceIls for patIents WIth systemIc lupus erythematosus. mcreased mterleukm-2 but not interleukin-4 mRNA", *Lupus* 3, 423-428.

[23] Viallard, J.E, Pellegrin, J.L., Ranchin, V., Schaaeverbeke, T, Dehais, J., Longy-Boursier, M., *et al.,* (1999) "Thl (IL-2, IFN-gamma) and Th2 (IL-IO IL-4) Cytokine product' on by peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE)", *Clin. Exp. Immunol.* 115, 189-195.

[24] Amital, H., Levi, Y, Blank, M., Barak, V., Langevitz, P., Afek, A, *et al.,* (1998) "Immunomodulation of murine experimental SLE-like disease by interferon-gamma", *Lupus* 7,445-454.

[25] Haas, C., Ryffel, B. and Le Hir, M. (1997) "IPN-gamma is essential for the development of autoimmune glomerulonephritis in MRLIpr mice", *J. Immunol.* 158,5484-5491.

[26] Peng, S.L.M.J. and Craft, J. (1997) "Roles of interferon-gamma and interleukin-4 in murine lupus", *J. Clin. Investig.* 99, 1936-1946.

[27] Jacob, e.O., van der Meide, P.H. and McDevitt, H.O. (1987) *"In vivo* treatment of (NZB X NZW)FI lupus-like nephritis with monoclonal antibody to gamma interferon", *J. Exp. Med.* 166, 798-803.

[28] Ozmen, L., Roman, D., Fountoulakis, M., Schmid, G., Ryffel, B. and Garotta, G. (1995) "Experimental therapy of systemic lupus erythematosus: the treatment of NZBfW mice with mouse soluble interferon-gamma receptor inhibits the onset of glomerulonephritis", *Eur. J. Immunol.* 25, 6-12.

[29] Davas, E.M., Tsirogianni, A., Kappou, I., Karamitsos, D., Economidou, I. and Dantis, P.e. (1999) "Serum IL-6, TNFalpha, p55 srTNFalpha, p75sr TNFalpha, srIL-2alpha levels and disease activity in systemic lupus erythematosus", *Clin. Rheumatol. 18,* 17-22.

[30] Segal, R, Dayan, M., Zinger, H. and Mozes, E. (1995) "Methotrexate treatment in murine experimental systemic lupus erythematosus (SLE); clinical benefits associated with cytokine manipulation", *Clin. Exp. Immunol.* 101,66-72.

[31] Dayan, M., Zinger, H., Kalush, E, Mor, G., Amir-Zaltzman, Y, Kohen, E, *et al.,* (1997) "The beneficial effects of treatment with tamoxifen and anti-oestradiol antibody on experimentalsystemic lupus erythematosus are associated with cytokine modulations", *Immunology* 90, 101-108.

[32] Segal, R, Dayan, M., Zinger, H. and Mozes, E. (2001) "Suppression of experimental systemic lupus erythematosus (SLE) in mice via TNF inhibition by an anti-TNFex monoclonal antibody and by pentoxiphyline", *Lupus* 10, 23-31.

[33] Yaswen, L., Kulkarni, A.B., Fredrickson, T, Mittleman, B., Schiffman, R., Payne, S., *et al.,* (1996) "Autoimmune manifestations in the transforming growth factor-beta I knockout mouse", *Blood* 87 1439-1445

[34] Ohtsuka,K, Gray, J.D., Stimmler, M.M., Toro, B. and Horwitz, D.A (1998) "Decreased production ofTGF-j3 by lymphocytes from patients with systemic lupus erythematosus" *J Immunol.* **160** 2539-2545.

[35] Kitamura, M., Suto, T., Yokoo, T, Shimizu, E and Fine, L.G. (1996) "Transforming growth factor-beta is the predominant paracrine mhIbItoro f maop hagecyd kinesyn theSIspro duced bygomeruaI mesenglal cells, *J. Immunol.* 156,2964-2971.

[36] EIaJ E., Zinger, H., Nyska, A. and Mozes, E (2000) PreventIon of systemIc lupus erythematosus- IIke dIsease in (NZB xNZW)FI mIce b" h CDRI d CDR3 b d d f h Y treat.mg w.t - an - ase peptI es o a pat ogemc autoantIbody, *J. Clm. Immunol.* .20,268-278

[37] ":Ioch, M.K., Alexa?er, A.L., PIppen, M.N., Isetsky, D.S. and GIlkson, G.S. (1997) Molecular propertIes of antI-DNA mduced m preau,01mmune ^{NZB}fW mIce by ImmumzatIon WIth bactena D.NA ,*J. Immuol.* 158, 4500-:-4506 ..

[38] TIllman, D., Nam-, Tsyr, J., HIll, R and MarIOn, T. (1992) Both IgM and IgG anJ-DNA atIbodIes are the prou;,ts of clonally selective B cell stimulatIOn m (NZBxNZW)FI mIce, *J. Exp. Med.* 176,761-779.

[39] Jouanne, C., Avrameas, S. and Payelle-Brogard, B. (1999) "A peptide derived from a polyreactive monoclonal anti-DNA natural antibody can modulate lupus development in (NZBXNZW)FI mice", *Immunology* 96,333-339.

[40] Kaliyaperumal, A., Michaels, M.A. and Datta, S.K (1999) "Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: tolerance spreading impairs pathogenic function of autoimmune T and B cells", *J. Immunol. 162,* 5775-5783.

[41] Gaynor, B., Putterman, e., Valadon, P., Spatz, L., Scharff, M.D. and Diamond, B. (1997) "Peptide inhibition of glomerular deposition of an anti-DNA antibody", *Proc. Natl Acad. Sci. USA* 94, 1955-1960.

[42] Singh, R.R., Ebling, EM., Sercarz, E.E and Hahn, RH. (1995) "Immune tolerance to autoantibody-derived peptides delays development of autoimmunity in murine lupus", *J. Clin. Investig.* 96, 2990-2996.

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Prevention of Systemic Lupus Erythematosus-Like Disease in (NZBxNZW)F1 Mice by Treating with CDR1- and CDR3-Based Peptides of a Pathogenic Autoantibody

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Two peptides based on the complementarity-determining regions (CDR) of a pathogenic murine anti-DNA antibody were employed in an attempt to prevent the spontaneous systemic lupus erythematosus (SLE)-like disease of (NZBxNZW)F1 mice. Female mice, at the age of 2 months, were injected with either the CDR1- or the CDR3-based peptides (pCDR1, pCDR3) subcutaneously or intravenously in aqueous solution for a total of 8–10 treatments. A reduction was observed in the total and pathogenic IgG2a and IgG3 anti-DNA antibody titers in the CDR-treated groups. Treatment reduced the number of mice that developed proteinuria and immune complex deposits in their kidneys. The severity of renal pathology was significantly reduced in the pCDR3 ($P < 0.02$) and pCDR1 ($P \leq 0.05$) treated mice. Thus, both CDR-based peptides administered in aqueous solution were capable of preventing the SLE-like disease in (NZBxNZW)F1 mice, although the beneficial effects of pCDR3 appeared to be more pronounced than those of pCDR1 in the treated mice.

KEY WORDS: SLE-like models; (NZBxNZW)F1 mice; CDR-based peptide; immunomodulation; disease prevention.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease and its complexity provides many challenges. There are several animal models for this disease, most of which are genetically based, and the mouse strains develop spontaneously SLE-like disease. (NZBxNZW)F1 mice develop a lupus-like syndrome that is manifested mainly in the production of high anti-DNA antibody titers and a kidney disease, which can be detected already by the age of 4 months, and leads to their death around the age of 10–12 months. Other SLE-prone mice include MRL lpr/lpr, BXSB, and Palmerstone-North (PN) (1, 2).

The induction of a lupus-like disease in non-genetically susceptible mouse strains has been previously reported by our laboratory (3). The model originally had been based on immunization of mice with a human monoclonal anti-DNA antibody, bearing a major idiotype namely 16/6 Id. A few weeks after immunization, mice develop anti-DNA and other antinuclear antibodies; anti-idiotypic antibodies; general clinical manifestations, including high erythrocyte sedimentation rate, leukopenia, and thrombocytopenia; and within 4 months from immunization the mice develop deteriorating kidney disease with immune complex deposits and sclerosis (3). Similar observations were demonstrated when disease was induced with a monoclonal murine anti-DNA antibody with the 16/6 Id, namely MAb 5G12 (4).

Two peptides based on the complementarity-determining regions (CDR), CDR1 and CDR3, of the murine anti-DNA antibody were designed and synthesized (5). We showed previously that injection of pCDR1 and pCDR3 in aqueous solution could inhibit the proliferative responses of lymph node cells of BALB/c and SJL strains, respectively, that were primed with either the pathogenic monoclonal anti-DNA antibodies of either murine (5G12) or human (16/6 Id) origin (5).

The murine monoclonal antibody 5G12 was shown to be highly homologous to anti-DNA antibodies isolated from (NZBxNZW)F1 (6, 7). Hence, it was of interest to attempt immunomodulation of the SLE-like disease in the SLE prone mice (NZBxNZW)F1 with the CDR-based peptides (pCDR1 and pCDR3). We report here that both peptides, given in phosphate-buffered saline

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(PBS), were capable of preventing disease development, however the beneficial effects of pCDR3 appeared to be more pronounced than those of pCDR1 in these mice.

MATERIALS AND METHODS

Mice

Eight- to ten-week-old female (NZBxNZW)F1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under standard conditions in the animal facility of the Weizmann Institute of Science.

Peptides

The CDR-based peptides of the murine anti-DNA antibody 5G12 were used. The CDR1-based peptide TGYYMQWVKQSPEKSLEWIG (pDCR1) and the CDR3-based peptide YYCARFLWEPYAMDYWGQGS (pCDR3) (the CDRs are underlined) were prepared with an automated synthesizer (Applied Biosystems, model 430A, Germany) using the company's protocols for *t*-butyloxycabonyl (*t*-BOC) strategy (8, 9). The reversed pCDR1 sequence peptide (GIWELSKEPSQKVWQ-MYYGT), which was shown to bind efficiently to the same MHC class II as pCDR1 but did not trigger T-cell responses (Eilat *et al.*, unpublished results), was synthesized as above and used as control for pCDR1. An analogue of pCDR3 (YYCARFLWEPYAN$_{\text{ORLEUCIN}}$DYWGQGS), where methionine was substituted by Norleucin was less efficient than pCDR3 in immunomodulating experimental SLE induced by the 16/6 Id (Brosh *et al.*, unpublished results) and was employed in the present study for comparison with pCDR3.

ELISA for DNA

For identification of anti-DNA antibodies, 96 wells Maxisorb microtiter plates (Nunc, Denmark) were coated with 50 μl/well of 10 μg/ml methylated bovine serum albumin (BSA) (Sigma, St. Louis, MO), or with 100 μl/well poly-L-lysine (5 μg/ml) (Sigma). The plates were then washed and coated with 50 μl of 10 μg/ml of denatured (boiled for 15 min and cooled rapidly on ice) calf thymus DNA (Sigma), or 100 μl/well of lamda phage dsDNA (Boehringer, Mannheim) (5 μg/ml). Thereafter, the plates were blocked with 1% ovalbumin (Sigma) in PBS, and the sera of the mice, diluted serially from 1:10 to 1:10^8, were incubated for 90 min. Plates were then washed and incubated for 60 min with goat anti-mouse IgG (gamma chain specific) conjugated to horseradish peroxidase (Jackson Immuno Research, West Grove, PA). Results of assays to determine ssDNA and dsDNA were compared and found to be similar. For the determination of the Ig isotypes of the antibodies assayed horseradish peroxidase-labeled goat anti-mouse IgG1 (γ1 chain specific), IgG2a, and IgG2b ($\gamma 2\alpha$, $\gamma 2\beta$chain specific) or IgG3 (γ3 chain specific) antibodies (Southern Biotechnology Associates, Birmingham, AL), were used. Following washing, plates were incubated with the substrate, ABTS {2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid); Sigma} and read using an enzyme-linked immunosorbent assay (ELISA) reader.

Detection of Proteinuria

Proteinuria was measured by a standard semiquantitative test, using an Albustix kit (Bayer Diagnostic, UK). Results were graded according to manufacturer as: negative; + = 0.3 g/liter, ++ = 1 g/liter, +++ = 3 g/liter, ++++ = $\geq$ 20 g/liter.

Immunohistology

Mice were sacrificed at the age of 8 months and kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections of 6 μm were air dried and fixed in acetone. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (Jackson Immuno Research, West Grove, PA). For the determination of the Ig isotypes of the antibodies assayed, FITC-conjugated goat anti-mouse IgG2a (γ2a chain specific) or IgG3 (γ3 chain specific) antibodies (Southern Biotechnology Associates, Inc. Birmingham, AL) were used. Specific staining was visualized using a fluorescence microscope. Immunohistology was evaluated with the technician blinded to whether mice belonged to control or experimental groups.

Pathological Evaluation

Mice were sacrificed and their kidneys were preserved frozen at −70°C, and then trimmed and routinely processed for light microscopy. Paraffin-embedded, 5-μm-thick sections were stained with hematoxylin and eosin (HE) and periodic acid Schiff (PAS). Lesions were described and scored where appropriate, using semiquantitative grading of five grades as follows: 0, no lesion; 1, minimal lesion; 2, mild lesion; 3, moderate lesion; and 4, severe lesion. Histopathology was evaluated with the pathologist blinded to whether mice belonged to control or experimental groups.

Statistical Analysis

To evaluate the significance of the difference between untreated and treated groups, the unpaired t-test and the nonparametric Wilcoxon rank test or Mann–Whitney test were used.

RESULTS

In order to find out whether treatment of (NZBxNZW)F1 mice with the CDR-based peptides at the age of 2 months, before disease manifestations are observed, will prevent the development of SLE in female (NZBxNZW)F1 mice, groups of the latter were injected with either pCDR1 or pCDR3 (250 μg/mouse or PBS subcutaneously) once a week for a total of 8 weeks. The mice were followed for anti-DNA antibody production and disease manifestations. A moderate reduction in anti-DNA antibody titers in treated groups, as represented in Fig. 1A and 1C in which sera taken from mice 1 month after the end of treatment, is seen. The reduction also is demonstrated in Fig. 2, in which the kinetics of total anti-DNA antibody production is shown in the pCDR3-treated group. The lower titers in the treated mice could be determined from the age of 5 months (1 month after the end of treatment) throughout the experiment. We were interested to examine whether this mild reduction in the total anti-DNA antibody could be attributed to one or more of the anti-DNA isotypes. Figure 1B and 1D demonstrate a moderate reduction in the pathogenic IgG2a and IgG3 in both the pCDR1-treated group and in the pCDR3-treated group. It should be noted that anti-DNA antibody titers of the IgG1 and IgG2b isotopes were very low in either the untreated or treated mice. This has been reproducible in the different experiments tested. As seen in Table I, significant reduction of proteinuria was observed in the groups treated with the CDR-based peptides. Thus, proteinuria of 8.8 g/liter was measured in the pCDR1-treated group and a more prominent reduction as low as 0.7 g/liter was observed in the pCDR3-treated group versus 20 g/liter in the PBS-treated group and 16 g/liter in the untreated group.

Table II demonstrates kidney analysis of the treated mice. As can be seen, treatment reduced the number of mice with immune complex deposits in their kidneys to 40% positive staining in the pCDR1-treated group and 25% in the pCDR3-treated group versus 80% in the PBS-treated group and 90% in the untreated group. Figure 3 demonstrates representative results of immune complex deposits in kidneys of either pCDR1- or pCDR3-treated mice. Kidney sections were stained with FITC conjugated to anti-IgG2a or IgG3 (the apparent pathogenic isotypes) antibodies. A reduction in deposition of both IgG2a and IgG3 anti-DNA antibodies could be observed. The reduction in immune complexes formation was more prominent in the pCDR3 treated group compared to the pCDR1 treated group.

The histopathological findings are presented in Table II and Fig. 4. Three components of renal pathological changes (i.e., glomerulonephritis) were evaluated: glomerulosclerosis, nephropathy, and interstitial lymphocytic infiltration. Glomerulosclerosis diagnosis was applied when the glomerular basement membrane appeared thickened. The process involved hyaline obliteration of the glomeruli, transforming them into acellular eosiophilic masses. This was particularly evident in PAS-stained sections. Nephropathy diagnosis was applied when the renal tubules appeared atrophic, regenerative, or basophilic, having dilated lumen with presence of proteinaceous casts. The term interstitial lymphocytic infiltration was applied to define the presence of scattered and discrete interstitial lymphocytic aggregation present at the medullary regions. Increased severity of glomerulosclerosis and nephropathy was accompanied by dissemination of lymphocytic infiltration within cortical and medullary regions. The mean severities of histopathological findings are presented in Table II. The incidence and in particular the severity grades of these parameters were significantly reduced in the groups treated with the peptides, as compared to the untreated group or to the group treated only with PBS. In particular, the severities of glomerulosclerosis and nephropathy, which are characteristic components of the spontaneous glomerulonephritis in the (NZBXNZW)F1 mice, were prominently reduced in the pCDR3 ($P < 0.02$) and pCDR1 ($P < 0.05$) treated mice (Fig. 4 and Table II).

It was of interest to evaluate the effects of an additional route of administration, namely intravenous (i.v.) treatment with pCDR1, on the clinical manifestations observed in (NZBxNZW)F1 mice. Therefore, mice were treated at the age of 3 months with i.v. injections of 100 μg/mouse twice a week for 4 weeks (or with reversed pCDR1 as control peptide). Treated mice showed significant reduction in proteinuria (1.9 g/liter vs. 6.3 g/liter in the untreated group or 8.4 g/liter in the control peptide treated mice) (Table III, experiment 1), whereas mild reduction in anti-DNA antibody titers was observed (Fig. 5A). Upon sacrifice the kidneys of the experimental mice were examined. As shown in Fig. 6, the immune complex deposits in the kidneys of the i.v. pCDR1-treated group (B) were less intense compared to the untreated (A) and the reversed pCDR1-treated groups (C).

The specificity of the beneficial effects of pCDR1 and pCDR3 was further tested. Mice were treated subcutane-

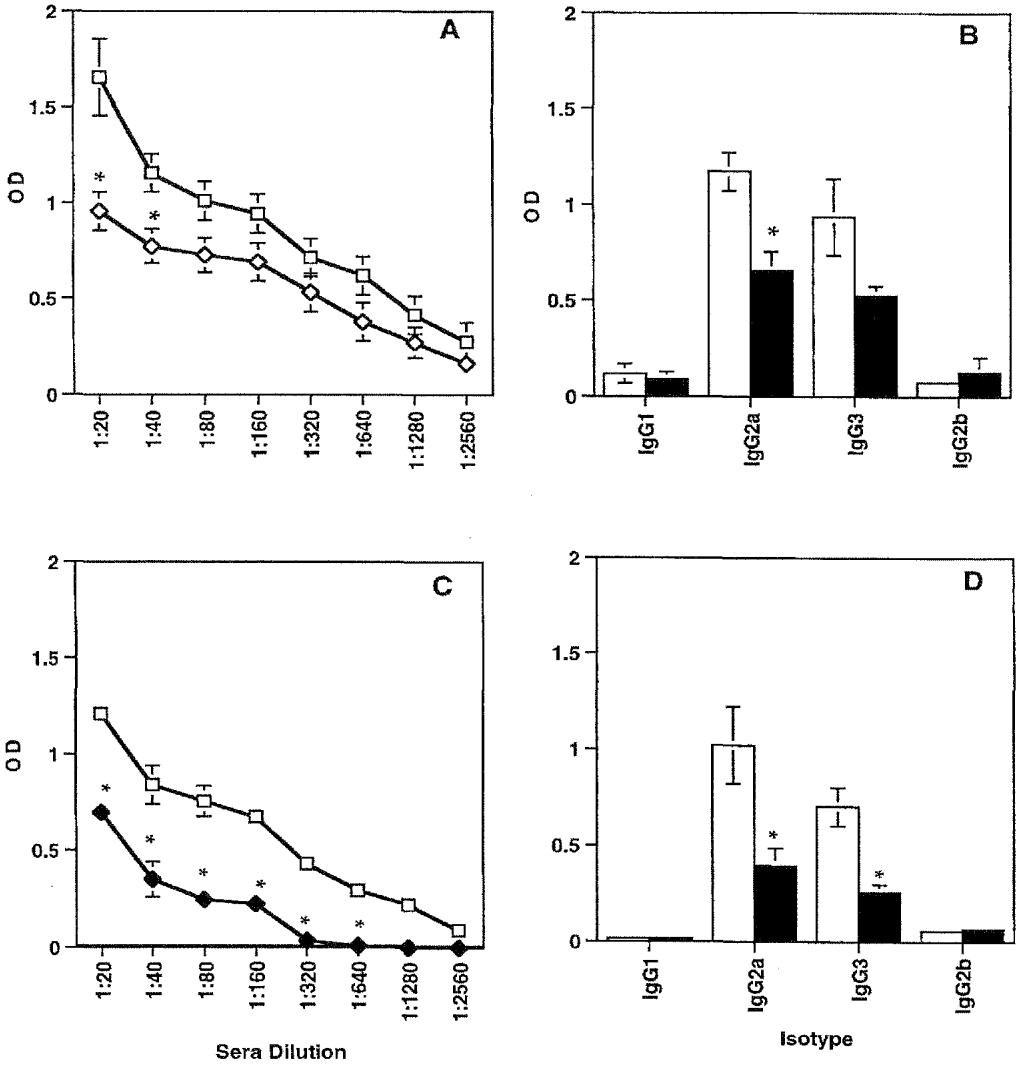

Fig. 1. Antibody levels in the sera of mice treated with the CDR-based peptides. Sera of individual (NZBxNZW)F1 mice (10 mice/group) (A) untreated (□), pCDR1 treated (◇), or (C) pCDR3 treated (◆), taken 1 month after the end of 8 weeks treatment were tested for antibody levels to DNA, by ELISA as described in Materials and Methods. Anti-DNA isotype levels in (NZBxNZW)F1 mice: untreated, white bars, treated mice, black bars, with (B) pCDR1 or (D) pCDR3 are presented at a dilution of 1:40. Results are expressed as mean OD ± SD of each mouse group. $^*P \leq 0.05$ as compared with the untreated groups. These results represent all bleedings.

ously (s.c.) at the age of 2 months with 250 μg/mouse of pCDR1 or control peptide (reversed pCDR1) twice a week for 4 weeks. A decrease in anti-DNA antibody titers was observed as shown in Fig. 5B. The decrease was determined for all IgG isotypes (IgG1, 2a, 2b, and 3; data not shown). Table III (experiment 2) shows that the treatment with pCDR1 significantly decreased proteinuria and immune complex deposits in kidneys (40% in the pCDR1-treated group as compared to 100% in the control group). Representative results of immune complex deposits in the kidneys are presented in Fig. 6, which demonstrates significant reduction in immune complex deposits in kidneys of mice treated with pCDR1 (D) compared to the untreated (A) or treated with control peptide groups (E).

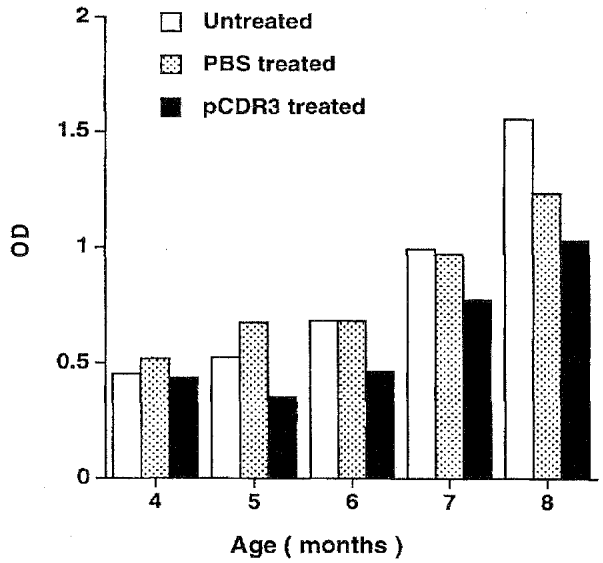

Fig. 2. Kinetics of anti-DNA antibody production in (NZBxNZW)F1 mice treated with pCDR3 at the age of 2 months. Mice were given weekly treatments with peptide pCDR3 from the age of 2 months during 8 weeks and bled monthly until the age of 8 months to determine anti-DNA antibody levels. Anti-DNA antibody levels were measured on pools of sera of each experimental group and presented at a dilution of 1:80.

We further compared the effect of treatment with pCDR3 to one of its analogues that was shown to inhibit pCDR3 proliferative responses *in vitro* and *ex vivo*. However, in long-term experiments of induced experimental SLE, this analogue failed to show significant protective effect (Brosh *et al.*, unpublished data). As seen in Fig. 7, moderate decreased anti-DNA antibody titers in the pCDR3 treated group compared to the analogue-treated group were determined. The decrease was manifested mainly in the pathogenic IgG2a isotype (data not shown). Table III (experiment 3) demonstrates that treatment with pCDR3 affected proteinuria (2.1 g/liter) compared to the untreated group (10.7 g/liter) and to the group treated with the pCDR3 analogue (7.2 g/liter). Immune complex deposits were dramatically decreased in the pCDR3-treated group compared to the pCDR3-based analogue and to the untreated group (40%, 85%, and 100% positive staining, respectively). As expected, the pCDR3-based analogue showed only mild beneficial effects, whereas the effects of pCDR3 were significant. Representative results are demonstrated in Fig. 6 and show that immune complex deposits are markedly reduced in the pCDR3-treated group (F) compared to the pCDR3-based analogue-treated group (G) and to the untreated group (A).

Table I. Treatment with CDR-Based Peptides Reduces the Levels of Protein in the Urine of (NZBxNZW)F1 Mice[a,b]

Treatment of (NZBxNZW)F1 mice	Proteinuria g/liter ± SD
Untreated	16.06 ± 7.8
pCDR1 s.c.	8.8 ± 9.1
pCDR3 s.c.	0.7 ± 0.12[c]
PBS s.c.	20 ± 0

[a] Proteinuria was always measured at about the same time of day, and all mice in an experimental cohort were tested together. Results are of mice at the age of 8 months.
[b] The results are representative of two experiments of treatment with pCDR1 and pCDR3.
[c] $P < 0.05$ as compared with the untreated group.

DISCUSSION

The main findings of the present report are that two peptides based on the CDR of a pathogenic anti-DNA antibody that induces experimental SLE in naive mice are capable of preventing the SLE-like disease in the SLE-prone (NZBxNZW)F1 mice. SLE is a complex disease that presents a challenge for immunomodulation, since its pathogenicity is not clear. The different animal models of SLE-like syndrome present various immune abnormalities that varies from one model to another. Thus, it is difficult to find a common feature that will provide the basis for treatment. Optimal treatment for SLE should consist of a specific agent. Therefore, we synthesized two peptides (pCDR1 and pCDR3) based on the CDR of a pathogenic autoantibody in an attempt to immunomodulate lupus-like disease. Injections of soluble pCDR1 or pCDR3 into BALB/c or SJL mice, respectively, inhibited the proliferative responses of lymph node-derived T cells of mice immunized with the pathogenic monoclonal human anti-DNA antibody with the 16/6 Id (5). We have shown further the efficacy of pCDR1 and pCDR3 in inhibiting the induction of the SLE-like disease induced by the 16/6 Id in BALB/c and SJL mice (Eilat, Brosh, and Mozes, unpublished data). The peptides used in the present study were synthesized based on the CDR of a monoclonal anti-DNA antibody, 5G12, which is highly homologous to an anti-DNA antibody isolated from (NZBxNZW)F1 mice, namely, F5-48 (6), and to a different anti-DNA antibody from the same mouse strain, namely, 17s.166 (7). The fact that the three anti-DNA antibodies (5G12, F5-48, 17s.166), which originated from C3H.SW and (NZBxNZW)F1 mice, are related might suggest a common background of the different SLE-like animal models, the genetically and induced disease, and could be attributed to consensus sequences of anti-DNA antibodies. Peptides derived from syngeneic different anti-DNA autoantibodies heavy

Table II. Treatment with the CDR-Based Peptides Reduces the Histopathological Findings and the Formation of Immune Complex Deposits in Kidneys of (NZBxNZW)F1 Mice[a]

Treatment	Histopathological findings[b]		Intensity of immune complex deposits[c]		Mice with immune complex deposits
			Grade	No. of animals	
Untreated	G	2.09 ± 0.9	+++	8	10/11 (90%)
	N	2.09 ± 1	++	1	
	I	1.9 ± 0.8	+	1	
			±	1	
pCDR1 s.c.	G	0.75 ± 1.3[d]	++	1	215 (40%)[d]
	N	0.8 ± 1.1[d]	+	1	
	I	1.2 ± 0.7	±	3	
pCDR s.c.	G	0[e]	++	2	2/8 (25%)[e]
	N	0[e]	±	6	
	I	0.87 ± 0.6			
PBS s.c.	G	2.4 ± 0.8	+++	4	4/5 (80%)
	N	2 ± 0.6	±	1	
	I	2.6 ± 0.5			

[a] Results are representative of two experiments of treatment with pCDR1 and pCDR3.
[b] Histopathological findings: paraffin embedded, 5-μm thick sections were stained with hematoxylin and cosin (HE) and periodic acid Schiff (PAS). Histopathological findings were evaluated in three components of renal pathological changes: G, glomerulosclerosis; N, nephropathy; I, interstitial lymphocytic infiltration. Microscopic evaluation: histopathological changes observed were graded on a scale of 0–4 and in ascending order, according to degree of severity as shown: 0, unremarkable; 1, minimal; 2, mild; 3, moderate; 4, marked change. For final evaluation, the mean value for severity of each observed lesion was calculated.
[c] Frozen cryostat sections were stained with FITC-labeled goat anti-mouse Ig (γ-chain specific). The intensity of the immune complex deposits was graded as follows: (−) or (±), no immune complex deposits or very low intensity; (+), minimal intensity; (++) moderate, and (+++) high intensity of immune complex deposits.
[d] $P < 0.05$ as compared with the untreated group.
[e] $P < 0.02$ as compared with the untreated group.

chain variable (V_H) regions were shown to specifically activate T cells of young (NZBxNZW)F1 mice. These peptides were suggested to have an important role in anti-DNA production and pathogenesis of murine lupus (10, 11).

A few approaches were used for the prevention of the SLE-like disease of (NZBxNZW)F1 mice. Thus, treatment of mice with the estrogen antagonist, tamoxifen, ameliorated lupus-like disease with a beneficial effect on clinical manifestations in this mouse strain and a significant decrease in pathogenic anti-DNA IgG3 antibody titers (Sthoeger *et al.*, unpublished data). Treatment of (NZBxNZW)F1 mice with methimazole (which down-regulates MHC class I) prevented the development of disease as assessed by a reduction of immune complex deposits in the kidneys of treated mice (12, 13). The *in vivo* elimination/inactivation of $CD4^+$T cells has been reported to be effective in the prevention and treatment of several murine models of autoimmune diseases (14, 15). However, a short course of anti-CD4 therapy in (NZBxNZW)F1 mice did not result in long-term suppression of autoimmunity (16). Early thymic radiation of (NZBxNZW)F1 mice at the age of 2 months led to decreased mortality and reduction in IgG3 anti-DNA antibodies, however, no reduction in immune complexes or anti-DNA antibodies was observed (17).

All the above treatment protocols had beneficial effects but are not specific to the SLE-like disease. Thus these treatments affect other functions of the immune system as well. Treatment with the CDR-based peptides is specific to lupus and was shown to efficiently ameliorate disease manifestation. Our study was not designed to determine survival of mice following treatment, since we sacrificed mice at age of 8 months to enable kidney disease analysis in both treated and untreated groups. However, our results led to reduction in anti-DNA antibodies especially in the pathogenic IgG2a and IgG3 subtypes. This reduction was observed in sera of both the pCDR1- and pCDR3-treated mice. We also observed a significant reduction in proteinuria in the mice treated with the CDR-based peptides, and most importantly, treatment with the CDR-based peptides prevented kidney damage in a significant number of the treated (NZBXNZW)F1 mice. Our results are in agreement with a recent report that injection of a pCDR3 peptide based on the CDR3 of an anti-DNA antibody from naive BALB/c mice to (NZBXNZW)F1 mice at the age of 2 months yielded delay of 50% in mortality rate and onset of proteinuria. The latter also induced IgG1 anti-DNA antibodies that were able to bind to the anti-DNA IgG2a subtype but not to polyclonal IgG2a (18). Further, inducing tolerance in

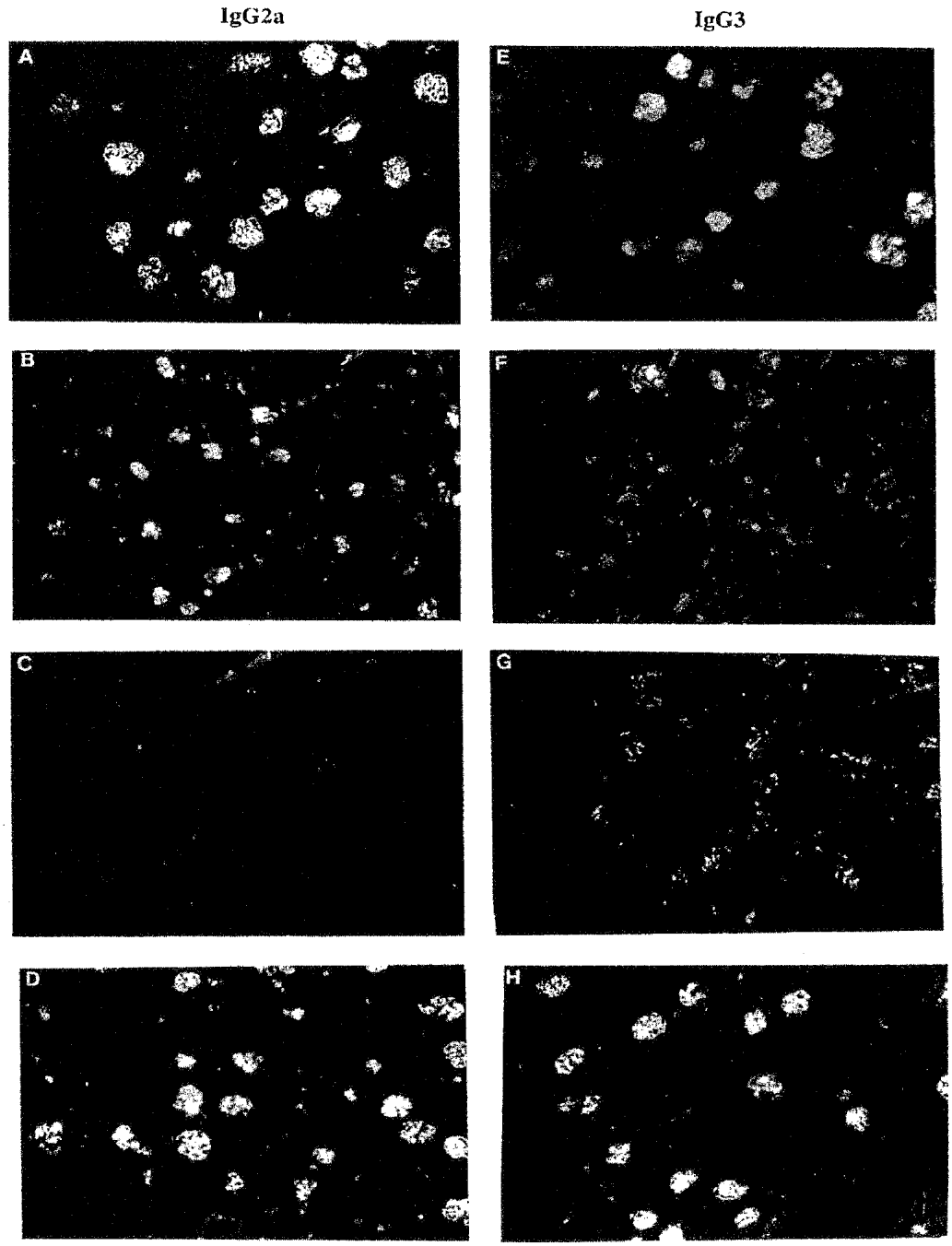

Fig. 3. Treatment with the CDR-based peptides reduces the incidence and severity of immune complex deposits in the kidneys of (NZBxNZW)F1 mice. Frozen kidney sections from untreated mice (A, E), control PBS-treated mice (D, H) pCDR1-treated mice (B, F), and pCDR3-treated mice (C, G) were fixed and stained as described (in Materials and Methods) for the detection of immune complex deposits of the IgG2a or IgG3 isotypes. The kidney sections shown are from a single animal within a group, but are representative of that group, as summarized in Table II (×100).

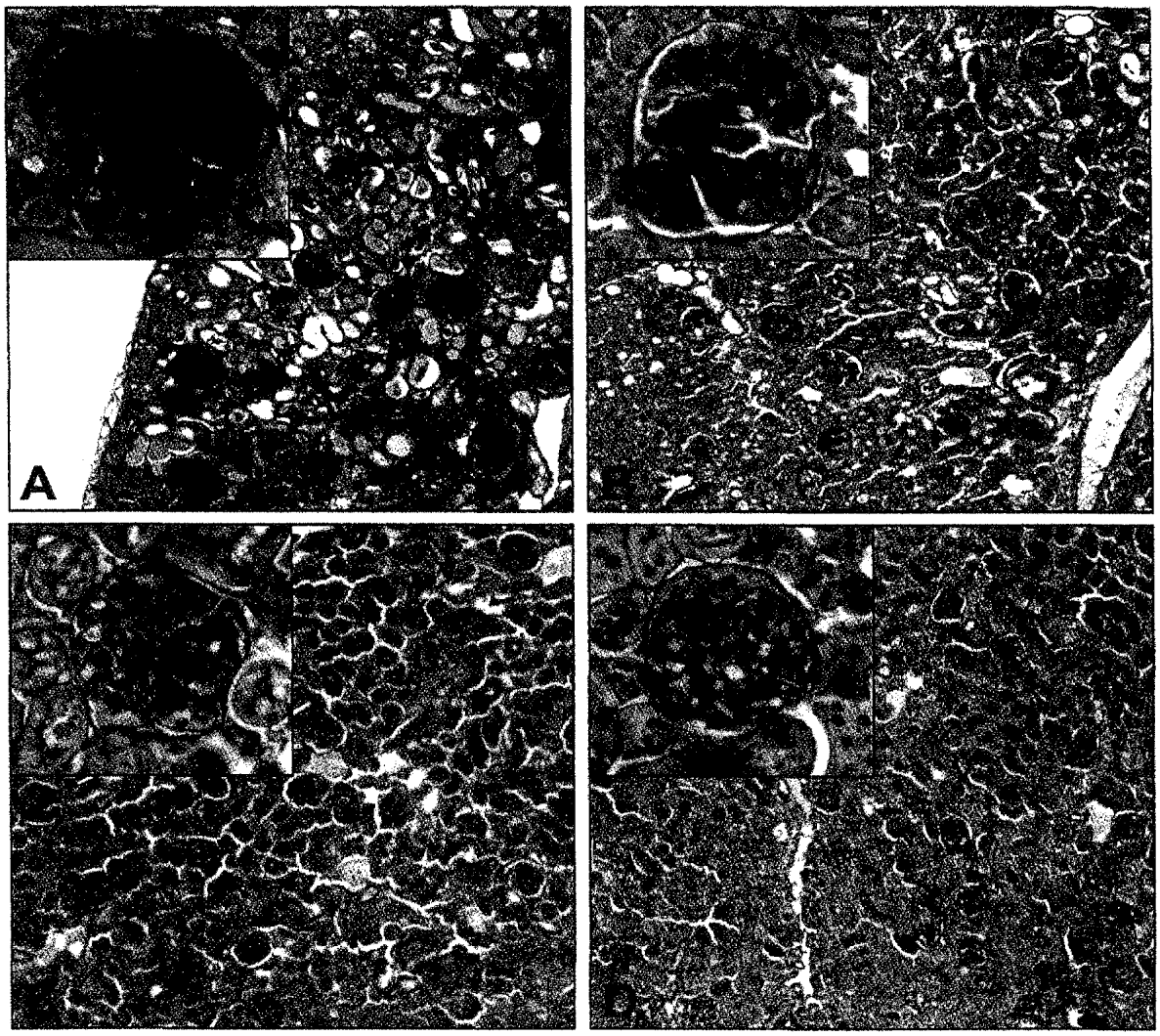

Fig. 4. Histopathological findings in (NZBxNZW)F1 mice treated with CDR-based peptides. (A) Representative histological renal section of a control (untreated) (NZBxNZW)F1 mouse. Note the marked degree of glomerulosclerosis consisting of marked thickening of the PAS+ glomerular basement membrane. (B) Representative histological renal section of a (NZBxNZW)F1 mouse treated for 8 weeks with PBS. Note the marked degree of glomerulosclerosis consisting of marked thickening of the PAS^+ glomerular basement membrane. (C) Representative histological renal section of a (NZBxNZW)F1 mouse treated for 8 weeks with pCDR1 peptide. Note no histopathological changes. The PAS^+ glomerular basement membranes are thin. (D) Representative histological renal section of a (NZBxNZW)F1 mouse treated for 8 weeks with pCDR3 peptide. Note no histopathological changes. The PAS^+ glomerular basement membranes are thin. PAS, 25×. Inset, 132×.

young (NZBXNZW)F1 mice by i.v. injections of peptides derived from the heavy chain variable (V_H) regions of anti-DNA autoantibodies postponed the development of the SLE-like disease in these mice (10, 19). Peptides based on a nephritogenic (R4A) anti-DNA antibody were shown to protect mice from renal deposition of anti-DNA antibodies (20). Thus, the reported, as well as our findings, support the crucial effect of heavy chain variable region-derived peptides in the modulation of murine lupus.

The mechanism underlying the immunomodulation of the CDR-based peptides has not been elucidated yet. Recent results in which the pCDR1 was used to prevent induction of experimental SLE in BALB/c mice indi-

Table III. Treatment with the CDR-Based Peptides Reduces the Intensity and Formation of Immune Complex Deposits in Kidneys of (NZBxNZW)F1 Mice[a]

Treatment	Proteinuria[b] g/liter ± SD	Intensity of immune complex deposits[c]	Mice with immune complex deposits
Experiment 1			
Untreated	6.3 ± 8.4	1.75 ± 0.43	100%
pCDR1 i.v.	1.9 ± 4.4[d]	0.5 ± 0.5[d]	50%
Reversed pCDR1 i.v.	8.4 ± 9.2	1.3 ± 0.7	86%
Experiment 2			
Untreated	11.6 ± 6.9	2.8 ± 0.41	100%
pCDR1 s.c.	3.9 ± 4.4[d]	0.8 ± 1[d]	40%
Reversed pCDR1 s.c.	9.3 ± 8.9	3 ± 0	100%
Experiment 3			
Untreated	10.7 ± 9.7	3.4 ± 0.48	100%
pCDR3 s.c.	2.1 ± 1.3[d]	0.8 ± 1[d]	40%
pCDR3 Analog s.c.	7.21 ± 8.5	1.9 ± 1.12	85%

[a] Results are representative of two experiments for each category.
[b] Proteinuria always was measured at about the same time of day, and all mice in an experimental cohort were tested together. Results are means of two to three measurements of mice at the age of 7–8 months.
[c] Frozen cryostat sections were stained with FITC-labeled goat anti-mouse Ig (γ-chain specific). The intensity of the immune complex deposits was graded on a scale of 0–4 in ascending order, according to degree of severity: (−, ±), 0; (+), 1; (++), 2; (+++), 3; (++++), 4. For final evaluation, the mean value was calculated.
[d] $P \leq 0.05$ as compared with the control groups.

cated that the pCDR1 treatment led to decreased levels of the Th1-type cytokines (IL-2, INFγ) that are required for the induction of experimental SLE (21), whereas secretion of the suppressive cytokine transforming growth factor-beta (TGF-β) was increased (Eilat *et al.*, unpublished data). These results could suggest that at least part of the beneficial effects of the CDR-based peptides is via immunomodulation of cytokine secretion. Further, down-regulation of the Th1-type cells could account for the observed reduction in the IgG2a and IgG3 (pathogenic) anti-DNA antibody levels. Down-regulation of epitope spreading might be one of the central means by which the CDR-based peptides immunomodulate SLE-associated responses. Indeed, epitope spreading was shown to play an important role in the induction and progression of lupus (10). Whether the CDR-based peptides inhibit autoreactive-determinant spreading via immunomodulation of cytokine production or by interference with other immune reactions is currently under investigation.

It is noteworthy that the CDR-based peptides immunomodulated SLE manifestations in the (NZBxNZW)F1 mice when given either intravenously or subcutaneously. Recent experiments have shown that the CDR-based peptides are protective when given orally, as well (Brosh *et al.*, unpublished results). It is thus possible that all the above administration routes (including i.v.) (22) lead to the production of the immunosuppressive cytokine TGF-β.

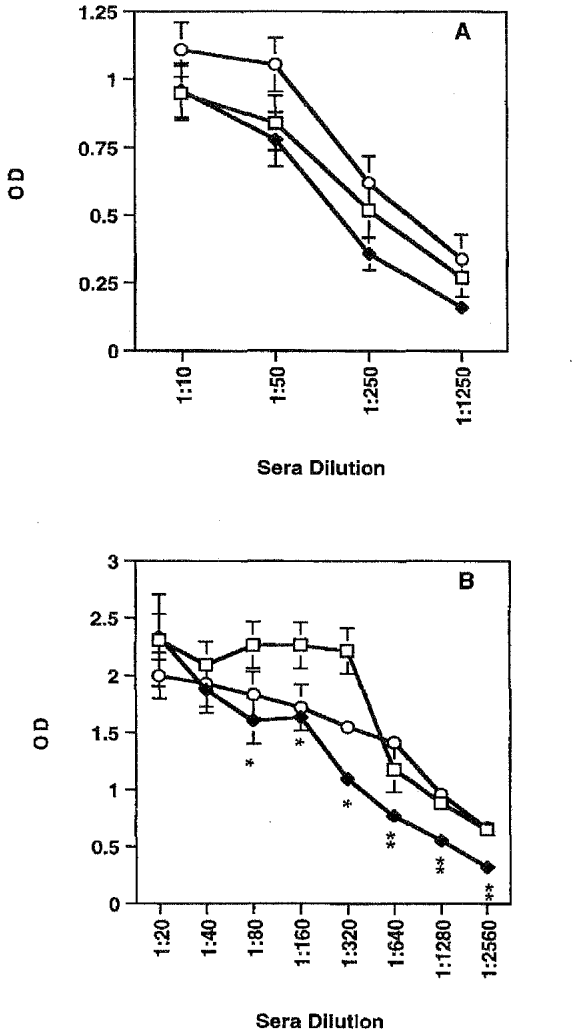

Fig. 5. Anti-DNA antibody levels in the sera of mice treated with CDR1 by different routes. Sera of individual (NZBxNZW)F1 mice (10 mice/group) injected with pCDR1 during 8 weeks, either i.v. (A) or s.c. (B), untreated (□), pCDR1 treated (◆), and reversed pCDR1 treated (○) taken 1 month after the end of the treatment were tested for antibody titers to DNA. Anti-DNA antibody levels were determined by ELISA. Results are expressed as mean OD ± SD of each mouse group. *$P < 0.05$ as compared to untreated mice; **$P < 0.05$ as compared to mice treated with the control peptide. These results represent all bleedings.

There is no specific treatment for SLE as yet; therefore, disease is treated with corticosteroids at initial stages, and when these fail, an immunosuppressive

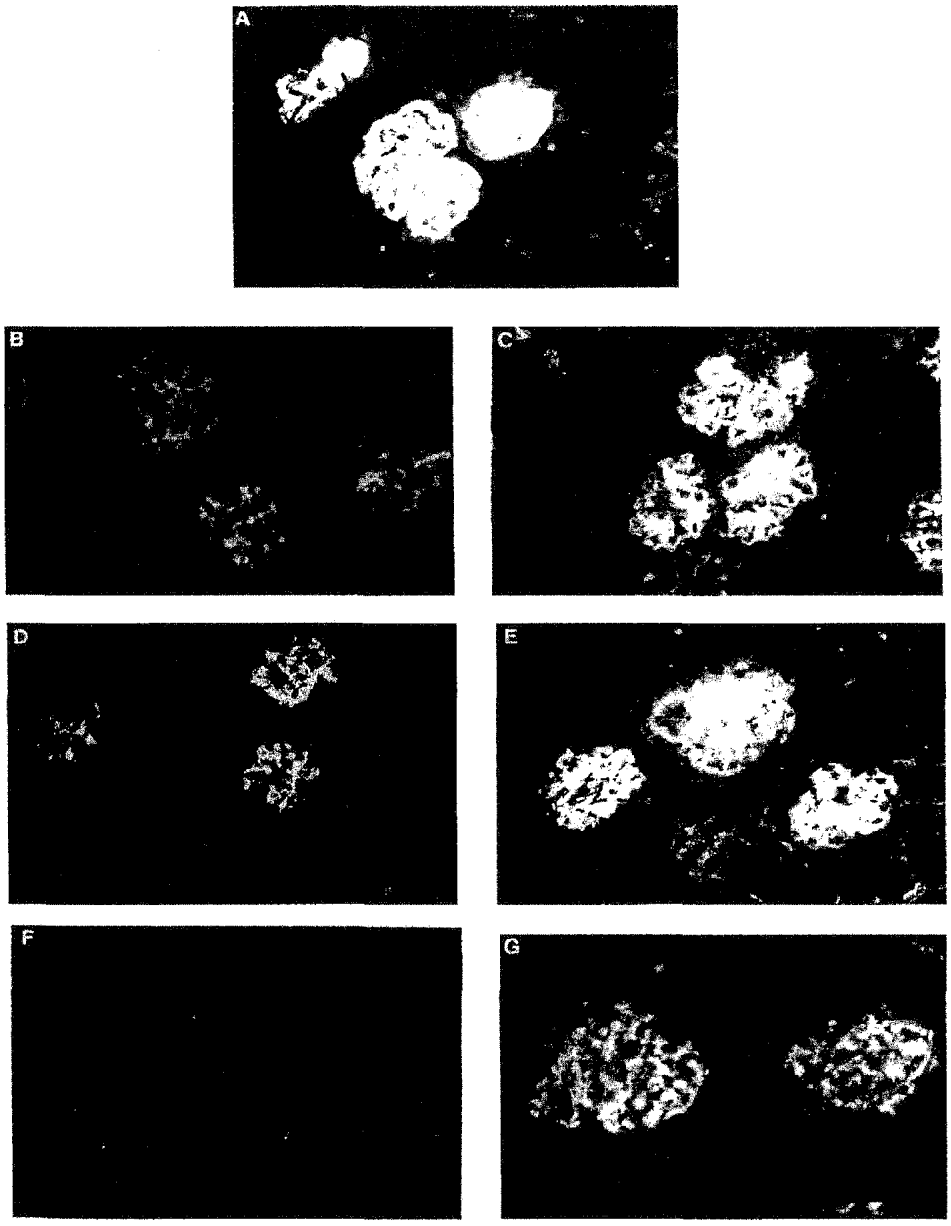

Fig. 6. Immunohistology of kidney sections of mice treated with the CDR-based peptides. (NZBxNZW)F1 mice were either untreated (A), pCDR1 treated i.v. (B), or s.c. (D), pCDR3 treated s.c. (F), reversed pCDR1 treated i.v. (C) or s.c. (E), and pCDR3-based analogue treated s.c. (G). At the age of 8 months mice were sacrificed and their kidneys removed and analyzed for the presence of immune complex deposits as described (×400).

treatment is initiated. The fact that peptides originated from an antibody of an inducible animal model can dramatically benefit a different spontaneous animal model is encouraging. Further testing of these peptides is needed to examine their effect on an established disease in (NZBxNZW)F1 mice. Preliminary results of treatment of (NZBxNZW)F1 mice at the stage that clinical manifestations are already observed suggest a

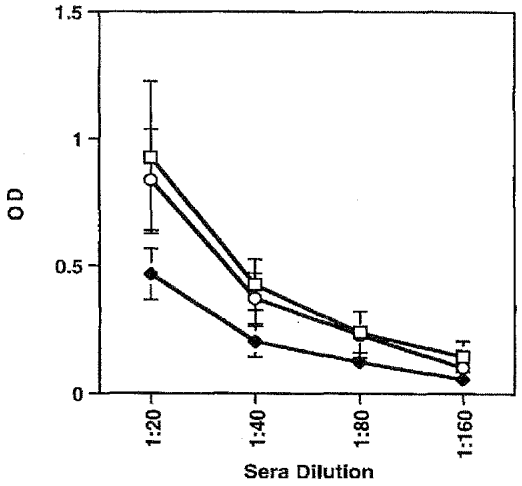

Fig. 7. Anti-DNA antibody levels in sera of (NZBxNZW)F1 mice treated with pCDR3 and its analogue. Sera of individual (NZBxNZW)F1 mice (10 mice/group): untreated (□), pCDR3 treated (◆), and pCDR3 analogue treated (○) taken 1 month after the end of 8 weeks treatment (weekly s.c. injections) were tested for antibodies to DNA. Anti-DNA antibody levels were determined by ELISA. Results are expressed as mean OD ± SD of each mouse group. These results represent all bleedings.

beneficial effect of the CDR-based peptides at this stage as well.

ACKNOWLEDGMENTS

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REFERENCES

1. Theofilopoulos AN: Murine models of lupus. *In* Systemic Lupus Erythematosus, G LR (ed). New York, Churchill Livingston, 1992, p 121–194
2. Mozes E: Experimental systemic lupus erythematosus: From a pathogenic autoantibody to immunomodulating peptides. *In* The Decade of Autoimmunity, Y Shoenfeld (ed). Amsterdam, The Netherlands, Elsevier Science B.V., 1999, pp 109–117
3. Mendlovic S, Brocke S, Shoenfeld Y, Ben Bassat M, Meshorer A, Bakimer R, Mozes E: Induction of a systemic lupus erythematosus-like disease in mice by a common human anti-DNA idiotype. Proc Natl Acad Sci USA 85:2260–2264, 1988
4. Waisman A, Mendelovic S, Ruiz JP, Zinger H, Meshorer A, Mozes E: The role of the 16/6 idiotype network in the induction and manifestation of systemic lupus erythematosus. Int Immunol 5:1293–1300, 1993
5. Waisman A, Ruiz PJ, Israeli E, Eilat E, Konen Waisman S, Zinger H, Dayan M, Mozes E: Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of a pathogenic anti-DNA monoclonal antibody. Proc Natl Acad Sci USA 94:4620–4625, 1997
6. Wloch MK, Alexander AL, Pippen AMN, Pisetsky DS, Gilkson GS: Molecular properties of anti-DNA induced in preautoimmune NZB/W mice by immunization with bacterial DNA. J Immunol 158:4500–4506, 1997
7. Tillman DM, Jou NT, Hill RJ, Marion TN: Both IgM and IgG anti-DNA antibodies are the products of clonally selective B cell stimulation in (NZBxNZW)F1 mice. J Exp Med 176:361–379, 1992
8. Kent SBH, Hood LE, Beilan H, Meister S, Geiser T: High yield chemical synthesis of biologically active peptides on an automated peptide synthesizer of novel design. *In* Peptides, U Ragnarsson (ed). Stockholm, Almqvist and Wiksell Int 1984, pp 185–188
9. Schnolzer M, Alewood PF, Kent SBH: *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int J Pept Protein Res 40:180–193, 1992
10. Singh RR, Hahn BH: Reciprocal T-B determinant spreading develops spontaneously in murine lupus: Implications for pathogenesis. Immunol Rev 164:201–208, 1998
11. Singh RR, Kumar V, Ebling FM, Southwood S, Sette A, Sercarz EE, Hahn BH: T cell determinants from autoantibodies to DNA can up-regulate autoimmunity in murine systemic lupus erythematosus. J Exp Med 181:2017–2027, 1995
12. Singer DS, Mozes E, Kirshner S, Kohn LD: Role of MHC class I molecules in autoimmune disease. Crit Rev Immunol 17:463–468, 1997
13. Mozes E, Zinger H, Kohn LD, Singer DS: Spontaneous autoimmune disease in (NZBxNZW)F1 mice is ameliorated by treatment with methimazole. J Clin Immunol 18:106–113, 1998
14. Wofsy D, Seaman WE: Successful treatment of autoimmunity in NZB/NZW F1 mice with monoclonal antibody to L3T4. J Exp Med 161:378–391, 1985.
15. Wofsy D: Administration of monoclonal anti-T cell antibodies retards murine lupus in BXSB mice. J Immunol 136:4554–4560, 1986
16. Connolly K, Roubinian JR, Wofsy D: Development of murine lupus in CD4-depleted NZB/NZW mice. Sustained inhibition of residual $CD4^+$ T cells is required to suppress autoimmunity. J Immunol 149:3083–3088, 1992
17. Erausquin C, Merino R, Izui S, Fernandez-Sueiro L, Saez F, Fernandez F, Rodriguez-Valverde VJM: Therapeutic effect of early thymic irradiation in (NZBxNZW)F1 mice, associated with a selective decrease in the levels of IgG3 and gp70-anti-gp70 immune complexes. Cell Immunol 161:207–212, 1995
18. Jouanne C, Avrameas S, Payelle-Brogard B: A peptide derived from a polyreactive monoclonal anti-DNA natural antibody can modulate lupus development in (NZBXNZW)F1 mice. Immunology 96:333–339, 1999
19. Singh RR, Ebling FM, Sercarz EE, Hahn BH: Immune tolerance to autoantibody-derived peptides delays development of autoimmunity in murine lupus. J Clin Invest 96:2990–2996, 1995
20. Gaynor B, Putterman C, Valadon P, Spatz L, Scharff M, Diamond B: Peptide inhibition of glomerular deposition of an anti-DNA antibody. Proc Natl Acad Sci USA 94:1955–1960, 1997
21. Segal R, Bermas BL, Dayan M, Kalush F, Shearer GM, Mozes E: Kinetics of cytokine production in experimental systemic lupus erythematosus: Involvement of T helper cell 1/T helper cell 2-type cytokines in disease. J Immunol 158:3009–3016, 1997
22. Williams ME, Caspar P, Oswald I, Sharma HK, Pankewycz O, Sher A, James SL: Vaccination routes that fail to elicit protective immunity against schistosoma mansoni induce the production of TGF-beta, which down-regulates macrophage antiparasitic activity. J Immunol 154:4693–4700, 1995

The mechanism by which a peptide based on complementarity-determining region-1 of a pathogenic anti-DNA auto-Ab ameliorates experimental systemic lupus erythematosus

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A peptide based on complementarity-determining region (CDR)-1 of a monoclonal murine anti-DNA Ab that bears the common idiotype, 16/6Id, was synthesized and characterized. The peptide, designated pCDR1, was found to be an immunodominant T-cell epitope in BALB/c mice. The CDR1-based peptide was shown to be capable of inhibiting the *in vivo* priming of BALB/c mice immunized with the peptide or with the whole anti-DNA 16/6Id$^+$ mAbs of either mouse or human origin. We show here that administration of pCDR1 (weekly, i.v., 100 μg/mouse) in aqueous solution for 5 weeks starting at the time of disease induction with the human 16/6Id prevented the development of clinical manifestations of experimental systemic lupus erythematosus (SLE). Further, 10 weekly injections of pCDR1 to BALB/c mice with an established experimental SLE down-regulated clinical manifestations of SLE (e.g., anti-DNA auto-Abs, leukopenia, proteinuria, immune complex deposits in the kidneys) in the treated mice. Prevention of SLE induction was shown to be associated mainly with a decrease in the levels of IL-2, INFγ, and the proinflammatory cytokine TNFα. On the other hand, the secretion of the immunosuppressive cytokine TGFβ was elevated. Amelioration of the clinical manifestations of an already established experimental SLE correlated with a dramatic decrease in TNFα secretion, elevated levels of TGFβ, and immunomodulation of the Th1 and Th2 type cytokines to levels close to those observed in healthy mice.

The induction of experimental systemic lupus erythematosus (SLE) has been previously reported in our laboratory and was achieved by using the human monoclonal anti-DNA Ab that bears the common idiotype, designated 16/6Id (1). This Ab could induce SLE in naive mice of different susceptible strains (2). The 16/6Id-induced disease resembles SLE in human and is manifested by high levels of auto-Abs, which include anti-DNA and antinuclear protein Abs as well as 16/6Id and anti-16/6Id specific Abs (1). The 16/6Id-immunized mice also develop lupus-associated clinical symptoms (e.g., leukopenia, proteinuria, and kidney damage). Experimental SLE can also be induced in mice after their immunization with either a murine anti-16/6Id mAb (3) or a murine anti-DNA 16/6Id+ mAb, 5G12 (4), suggesting the importance of the 16/6Id network in the disease. Furthermore, T-cell lines specific to the human anti-DNA 16/6Id$^+$ mAb were shown to be capable of inducing experimental SLE in syngeneic recipient mice indicating the role of T cells in the disease (5). Experimental SLE, although induced in mice that normally develop no symptoms of SLE, was found to share features with the SLE model of (NZBxNZW)F1 mice, which develop the disease spontaneously. Thus, sequencing of the variable regions coding for the heavy and light chains of anti-DNA mAb isolated from mice afflicted with experimental SLE show high homology with the variable regions of anti-DNA mAb isolated from (NZBxNZW)F1 mice (6).

Two peptides based on the sequences of the complementarity-determining regions (CDR) of the pathogenic murine monoclonal anti-DNA Ab (5G12) that bears the 16/6 Id were synthesized. pCDR1 and pCDR3 were shown to be immunodominant T-cell epitopes in BALB/c and SJL mouse strains, respectively, and induced a mild SLE-like disease in responder mice (7). Further, the CDR-based peptides inhibited the priming of lymph-node cells (LNC) of mice immunized with the same peptides or with the monoclonal anti-DNA 16/6Id$^+$ Abs of either mouse or human origin. The CDR1-based peptide was also shown to prevent auto-Ab production in BALB/c neonatal mice that were immunized later with either pCDR1 or the pathogenic auto-Ab (7).

In the present report, the ability of the CDR1-based peptide to immunomodulate SLE induced in BALB/c mice was tested. We show here that pCDR1 is capable of either preventing or treating an already established SLE-like disease. A decrease in Th1-type (IL-2, INFγ) cytokines was observed when mice were treated for experimental SLE prevention, whereas the amelioration of disease manifestations in the treatment protocol was associated with a pattern of Th1 and Th2 cytokines similar to that observed in healthy mice. A significant down-regulation of the proinflammatory cytokine TNFα and an up-regulated secretion of the immunosuppressive cytokine TGFβ was demonstrated in mice treated for either the prevention or immunomodulation of experimental SLE.

Materials and Methods

Mice. Mice of the BALB/c inbred strain were obtained from Olac (Bichester, U.K.). Female mice were used at the age of 8–10 weeks, unless specified otherwise.

Synthetic Peptides. The peptide based on the CDR1 TGYYMQWVKQSPEKSLEWIG (pCDR1; the CDR is underlined) of the murine monoclonal anti-DNA 16/6Id$^+$ auto-Ab (mAb 5G12; ref. 4), was prepared with an automated synthesizer (Applied Biosystems model 430A) by using the company's protocol for *t*-butyloxycarbonyl strategy (8). A peptide synthesized in the reversed order of pCDR1 (rev pCDR1) was used for control.

mAbs. The human anti-DNA mAb that bears the 16/6Id (IgG1/κ) was previously described (9, 10). The Ab was secreted by hybridoma cells that were grown in culture and was purified by using a protein G-Sepharose column (Pharmacia).

Abbreviations: CDR, complementarity-determining region; Id, idiotype; LNC, lymph node cells; pCDR1, peptide based on the sequence of CDR1; SLE, systemic lupus erythematosus.

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Immunization and Induction of Experimental SLE. To induce experimental SLE, mice were immunized with 1–2 μg of the human mAb 16/6Id and boosted 3 weeks later (1).

Treatment with the CDR1-Based Peptide. For prevention of experimental SLE, mice were given pCDR1 or the reversed pCDR1 (control peptide) i.v. (100 μg/mouse) concomitant with immunization and were injected weekly thereafter for 5 weeks. Treatment of an established disease had started 3.5 months after disease induction with the 16/6Id, when clinical manifestations were already observed. Mice received 10 weekly injections of the CDR1-based peptide given i.v. or s.c. (100 μg/mouse). Reversed pCDR1 was administered as control. Both prevention and treatment experiments were performed three times each.

Detection of SLE-Associated Clinical and Pathological Manifestations. Proteinuria was measured semiquantitatively by using Combistix kit (Ames Division, Bayer Diagnostics, Newbury, U.K.). White blood cells were counted after a 10-fold dilution of heparinized blood in distilled water containing 1% acetic acid (vol/vol). For immunohistology analysis, frozen kidney sections (6 μm) were fixed and stained with FITC-conjugated goat Abs to mouse IgG (γ-chain specific; Sigma).

ELISA. For measuring anti-DNA Abs, 96-well Maxisorb microtiter plates (Nunc) were coated with either methylated BSA or polyL-lysine (Sigma). The plates were then washed and coated with either 10 μg/ml of denatured calf thymus DNA (Sigma) or λ-phage double-stranded DNA (Boehringer Mannheim, 5 μg/ml). After incubation with different dilutions of sera, goat anti-mouse IgG (γ-chain specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch) was added to the plates, followed by the addition of the substrate, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). Results were read by using an ELISA reader. Results of assays to determine single- and double-stranded DNA were found to be similar. For the determination of 16/6Id-specific Abs, plates were coated with 10 μg/ml of human 16/6Id, and Abs to nuclear proteins were detected by using precoated plates (Diamedix, Miami). The assays were carried out as above.

Induction of Cytokines Production. Mice that were immunized with the human 16/6Id and either treated or not with the CDR1-based peptide were killed at different periods during or after treatment with pCDR1. Splenocytes and LNC were harvested and incubated (5×10^6/ml) in the presence of the 16/6Id. Supernatants were collected after 48 and 72 h.

Detection of Cytokines in Supernatants. Measurements of IL-2, -4, -10, INFγ, and TNFα were performed by ELISA by using the relevant standards, capture and detecting Abs (PharMingen) according to the manufacturer's instructions. For detection of TGFβ, plates were coated with recombinant human TGFβ1 sRII/Fc chimera (R & D Systems), and the second Ab used was the biotinylated anti-human TGFβ1 Ab (R & D).The substrate solution used was TMB color Reagent (Helix Diagnostics, West Sacramento, CA), and enzyme activity was evaluated by using 570- and 630-nm filters.

Detection of Intracellular Cytokines. Single-cell suspensions of LNC were exposed to a Cytoperm kit (Serotec) according to the company's protocol. Thereafter, cells were incubated with the appropriate anticytokine–FITC-conjugated Ab. Cells were assessed by a FACScan cytometer, and the data were analyzed by using LYSIS software (Becton Dickinson).

Statistical Analysis. Mann–Whitney and t tests were used for statistical analyses of the data.

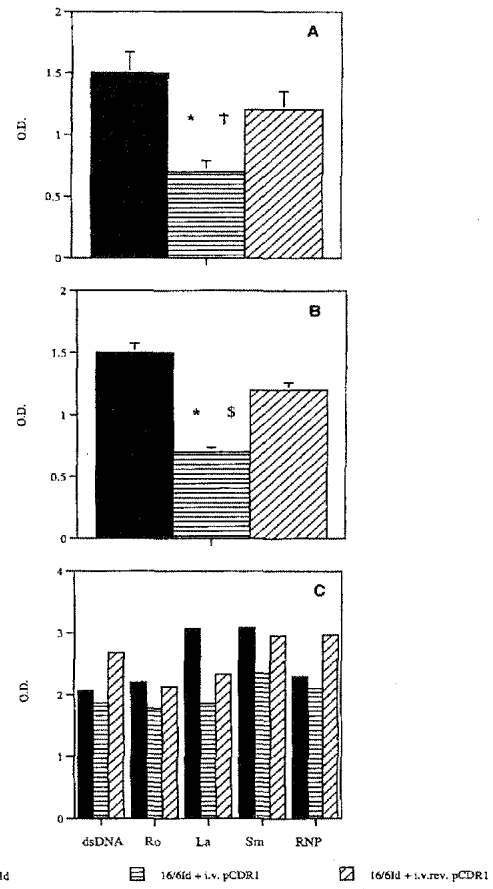

Fig. 1. Auto-Abs in BALB/c mice immunized with the 16/6Id. BALB/c mice (five mice per group) were immunized with 16/6 Id and concomitantly injected with pCDR1 or reversed pCDR1(100 μg/mouse i.v. once per week for 5 weeks) or were not treated. Results expressed as OD ± SD were obtained at the bleeding before sacrifice and represent all monthly bleedings. (*A*) Anti-DNA Ab titers measured on individual sera (dilution 1:1,000). (*B*) Anti-16/6Id Ab titers measured on individual sera (dilution 1:10,000). (*C*) Abs to nuclear antigens measured on pooled sera (dilution 1:100). OD levels of normal sera and of sera of mice that were injected only with pCDR1 were undetectable. The results are representatives of three experiments. (*A*) *, $P < 0.05$ as compared with reversed pCDR1-treated mice. †, $P < 0.01$ as compared with 16/6Id immunized untreated mice. (*B*) $, $P < 0.05$ as compared with 16/6Id immunized untreated or * 16/6Id immunized and reversed pCDR1-treated mice.

Results

Prevention of Experimental SLE. To find out whether the CDR1-based peptide is capable of preventing experimental SLE induced by the human anti-DNA 16/6Id, mice immunized with the latter were treated once per week for 5 weeks with pCDR1 (i.v. in PBS, 100 μg/mouse) or with a control peptide (reversed pCDR1), starting at the day of priming with 16/6Id. Fig. 1, which represents three similar experiments, demonstrates a decrease in the titer of anti-DNA (Fig. 1*A*), anti-16/6Id (Fig. 1*B*), and Abs to nuclear antigens (Fig. 1*C*) in the pCDR1-treated group compared with untreated or reversed pCDR1-treated groups. Table 1 shows that the clinical manifestations tested, namely leukopenia, proteinuria, and immune complex deposits in the kidneys, were also milder in the pCDR1-treated group of mice.

Table 1. The effect of treatment with pCDR1 on the clinical manifestations of experimental SLE

Immunization and treatment	WBC (mean ± SD)	Proteinuria (mean g/l ± SD)	Mean intensity of immune complex deposits ± SD
16/6 Id	2760 ± 391	1.4 ± 0.9	1.1 ± 0.2
16/6Id + reversed pCDR1	3220 ± 311	1.8 ± 1	0.88 ± 0.2
16/6Id + pCDR1	5950 ± 420*†	0.475 ± 0.35‡§	0.37 ± 0.1¶
pCDR1 only	5750 ± 208	0.225 ± 0.15	0.16 ± 0.1
Normal mice	5340 ± 313	0.18 ± 0.16	0.09 ± 0.09

BALB/c mice were immunized with 16/6Id and concomitantly injected with pCDR1 or reversed pCDR1 100 μg/mouse i.v. once per week for 5 weeks. Mice were followed for 8 months. Results of leukopenia and proteinuria were obtained 7 months after immunization and are representative of 3 experiments and of measurements performed at different time points. Results of immune complex deposits were evaluated as follows: 0 = no lesions or minimal lesions; 1 = moderate lesions; 2 = severe lesions. Kidney analyses were performed at death.
*, $P < 0.01$, ‡ and ¶, $P < 0.03$ compared to 16/6Id immunized mice that were not treated. †, $P < 0.01$ and §, $P < 0.03$ compared to 16/6Id immunized and reversed pCDR1 treated mice, respectively.

Fig. 2 demonstrates representative kidney sections of experimental mice. It can be seen in the figure that administration of pCDR1 prevented the formation of immune complex deposits in the kidneys. The effect of pCDR1 is specific because the control peptide, reversed pCDR1, did not affect specifically the auto-Ab titer (Fig. 1) and clinical manifestations, including kidney damage (Table 1, Fig. 2).

Treatment of Experimental SLE. It was of interest to find out whether the CDR1-based peptide is capable of down-regulating manifestations of experimental SLE when clinical symptoms are already observed. To this end, mice were immunized and boosted with the 16/6 Id and were followed for 3.5 months until clinical manifestations of the disease occurred. Groups of mice were then treated with 100 μg/mouse of pCDR1 administered either i.v. or s.c. once per week for 10 weeks. Fig. 3 demonstrates a significant decrease in the anti-DNA Ab titers in the pCDR1-treated groups (either i.v. or s.c., $P < 0.01$). Significantly reduced clinical manifestations (leukopenia, proteinuria, and immune complex deposits in the kidneys) were observed in the i.v.-treated group and to a lesser extent in the s.c.-treated group compared with the untreated group, as can be seen in Table 2. Fig. 4 represents immunohistology results of kidney sections of the different experimental groups. Both i.v. and s.c. administration protocols diminished the immune complex deposits in the pCDR1-treated groups. No such amelioration could be observed in the group of mice treated with the control peptide, the reversed pCDR1 (Table 2, Fig. 4). These results were reproducible in three independent experiments.

pCDR1 Down-Regulates Experimental SLE by Immunomodulating the Cytokine Profile. Because cytokines were shown to play a major role in the pathogenesis of experimental SLE (11), it was of interest to find out whether treatment with pCDR1 affects the cytokine profile of the treated mice. Hence, BALB/c mice that were injected with 16/6 Id and treated with pCDR1 were killed monthly, and their LNC were stained for intracellular cytokines. Table 3 represents results that were obtained 1 month after booster injection with the 16/6Id. A decrease in specific staining for IL-2 and INFγ could be observed in lymph node cells of mice of the pCDR1-treated group. A similar decrease could be observed 1 month later (data not shown). As can be seen in the table, no detectable changes could be observed in lymph node cells stained for IL-4 and -10. Matching results were obtained in a second independent experiment. We also examined secreted cytokines in supernatants of LNC and spleens of the experi-

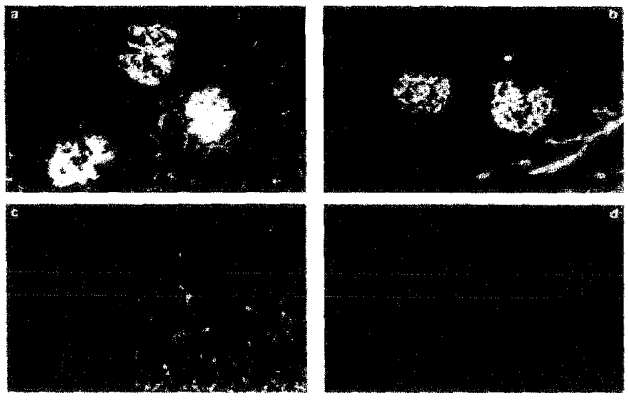

Fig. 2. Immunohistology of kidney sections of BALB/c mice that were treated with pCDR1 for prevention of experimental SLE. (*a*) 16/6Id-immunized mice; (*b*) mice immunized with 16/6 Id and concomitantly injected with reversed pCDR1; (*c*) 16/6Id-injected mice that were treated with pCDR1; (*d*) nonimmunized mice treated with pCDR1. Mice were killed 8 months after disease induction and their kidneys removed and analyzed for the presence of immune complex deposits as described in *Materials and Methods* (×400).

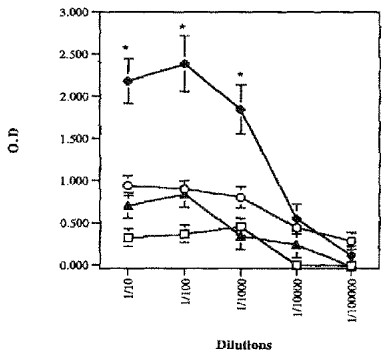

Fig. 3. Anti-DNA Abs in sera of SLE-afflicted BALB/c mice that were treated with pCDR1. BALB/c mice (20 mice/group) were immunized and boosted with 16/6Id. Later (3.5 months) mice were treated with pCDR1 i.v. or s.c., 100 μg/mouse once per week for 10 weeks. ◆, 16/6Id immunized; ○, 16/6Id immunized and treated with pCDR1 i.v.; ▲, 16/6Id immunized and treated with pCDR1 s.c.; □, sera of normal mice. Results expressed as OD ± SD were obtained at bleeding before death and represent all previous bleedings. Results were reproducible in three independent experiments. *, $P < 0.01$ as compared with both pCDR1-treated groups.

Table 2. Therapeutic effects of treatment with pCDR1 on the clinical manifestations of an established experimental SLE

Group	Immunization and treatment	WBC (mean ± SD)	Proteinuria (mean g/l ± SD)	Mean intensity of immune complex deposits ± SD
A	16/6Id	2870 ± 494	1.53 ± 1	1.5 ± 0.2
B	16/6Id + i.v. reversed pCDR1	3120 ± 701	1.67 ± 1.1	1.5 ± 0.5
C	16/6Id + i.v. pCDR1	6200 ± 490*	0.35 ± 0.37†	0.5 ± 0.3‡
D	16/6Id + s.c. pCDR1	5070 ± 625§	0.58 ± 0.36¶	0.66 ± 0.3**
E	Normal mice	7420 ± 511	0.06 ± 0.13	0

BALB/c mice were immunized with 16/6Id. Three and one half months after disease induction, they were treated with either pCDR1 (s.c. or i.v.) or reversed pCDR1 (i.v.) once per week for 10 weeks. Intensity of immune complex deposits were evaluated as described for Table 1. The above results were obtained at sacrifice (about 2 months after treatment had stopped).
*, $P < 0.01$ compared to group A and $P < 0.05$ compared to group B. ‡, $P < 0.01$ compared to group A and $P < 0.05$ compared to group B. †, $P < 0.02$ compared to groups A and B. ¶, $P < 0.02$ compared to groups A and B. ‡, $P < 0.03$ compared to group A. **, $P < 0.03$ compared to group A.

mental mice. Table 4 demonstrates the cytokine levels in the supernatants of 16/6 Id-stimulated LNC, about 2 weeks after the end of treatment (1 month after boosting with 16/6Id). As can be seen in the table, levels of proinflammatory cytokine TNFα as well as of IL-2 and INFγ were significantly lower in the group of pCDR1-treated mice, whereas secretion of the immunosuppressive cytokine TGFβ was elevated in these mice. The levels of IL-4 were below the detection sensitivity of the assay, whereas levels of IL-10 that were low in the 16/6Id-injected mice at the time of the assay were higher in the pCDR1-treated mice (260 pg/ml in supernatants of the pCDR1-treated mice compared with 120 and 140 pg/ml in 16/6Id-injected mice that were not treated or were treated with the control-reversed pCDR1, respectively). At a more progressed stage of the disease, when high levels of IL-10 were detected in supernatants of the SLE-afflicted mice (11), the concentration of the latter cytokine was lower in the pCDR1-treated mice and similar to that determined in normal mice (200 pg/ml in supernatants of pCDR1-treated mice as compared with 500 pg/ml and 460 pg/ml in supernatant of 16/6Id-injected and nontreated mice or reversed pCDR1-treated mice, respectively). Similar results were obtained when supernatants of splenocytes of the same mice were tested after their stimulation with the 16/6Id (data not shown).

We wanted to find out whether treatment with pCDR1 of mice with an already established experimental SLE affects the cytokine pattern as well. To this end, BALB/c mice with 16/6 Id-induced experimental SLE were treated after their clinical symptoms were observed. Two mice were killed monthly, and cytokines secreted by their LNC and spleen cells were assessed. Fig. 5 demonstrates the levels of cytokine secretion from spleens, at the end of treatment (about 6 months after disease induction), in comparison to cytokines in spleens of normal mice. A striking reduction in the levels of TNFα secreted by splenocytes of 16/6Id-immunized mice that either were not treated or were treated with the reversed CDR1-based peptide could be observed in supernatants of splenocytes of pCDR1-treated mice (either i.v. or s.c.). Both i.v. and s.c. treatment protocols increased significantly the levels of secreted TGFβ (Fig. 5). We have previously shown that at a progressed stage of the disease, the levels of secreted IL-2, INFγ, and IL-4 in the SLE-afflicted mice were lower than in healthy controls (11). Indeed, as can be seen in Fig. 5, treatment with pCDR1 (either i.v. or s.c.) resulted in the secretion of levels of the latter cytokines that are comparable to those determined in splenocytes of healthy mice. It is also shown in the figure that the secretion of IL-10 was immunomodulated by the treatment with pCDR1 to levels that are not substantially different from those of the normal mice. Similar results were obtained when cytokine secretion was measured in supernatants of LNC of mice of the different groups (data not shown). Thus, the beneficial effects of treatment with the CDR1-based peptide are associated mainly with the down-regulation of the proinflammatory cytokine TNFα that was shown to play a pathogenic role in SLE (11) and with an up-regulation in the secretion of the immunosuppressive cytokine TGFβ. The i.v. and s.c. treatment protocols had similar

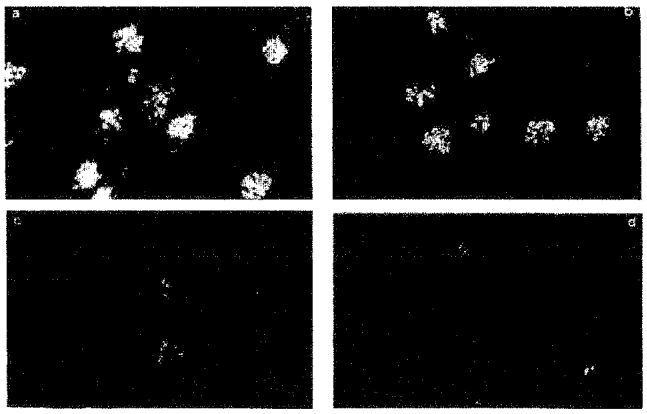

Fig. 4. Immunohistology of kidney sections of BALB/c mice treated with pCDR1 after clinical symptoms were observed. (*a*) 16/6Id immunized mice; (*b*) 16/6Id immunized mice treated with reversed pCDR1 i.v.; (*c*) 16/6Id immunized mice treated with pCDR1 s.c.; (*d*) 16/6Id immunized mice treated with pCDR1 i.v. Mice were killed 8 months after disease induction and their kidneys removed and analyzed for the presence of immune complex deposits (×20).

Table 3. Cytokine profile in BALB/c mice that were treated with pCDR1 for the prevention of experimental SLE

Cytokine	Intracellular staining: 16/6Id,* %	16/6Id + pCDR1, %	16/6Id + reversed pCDR1, %
IL-2	100	47	115
INFγ	100	56	81
IL-4	100	100	98
IL-10	100	99	98

Cytokine profile was determined by intracellular staining of lymph node cells (see *Materials and Methods*) of mice killed 1 month after booster injection with the 16/6Id (≈2 weeks after treatment). The results are representative of two experiments (5–12% variations were observed between experiments).
*Staining of lymph node cells of mice immunized with 16/6Id was considered as 100% (100% stained cells = 2,650, 2,950, 2,300, and 1,550 for IL-2, INFγ, IL-4, and IL-10, respectively).

Table 4. Cytokine profile in BALB/c mice that were treated with pCDR1 for the prevention of experimental SLE

Cytokine	Cytokine secretion		
	16/6Id, pg/ml	16/6Id + pCDR1, pg/ml	16/6Id + reversed pCDR1, pg/ml
IL-2	800 ± 141	<60	806 ± 137
INFγ	9,000 ± 816	267 ± 94	8,000 ± 2,160
TNFα	470 ± 30	<20	590 ± 10
TGFβ	2,350 ± 150	4,300 ± 300	2,900 ± 100

Secretion of cytokines was determined by ELISA (see *Materials and Methods*) of supernatants of LNC stimulated with the 16/6Id. Results are of mice killed 1 month after booster injection with the 16/6Id (≈2 weeks after treatment). The results are representative of two experiments.

effects on the clinical manifestations as well as on cytokine secretion.

Discussion

The main findings of the present report are that pCDR1 is capable either of preventing experimental SLE or of treating an already established SLE-like disease. The immunomodulation of disease manifestations was shown to be associated mainly with a significant down-regulation of the proinflammatory cytokine TNFα and with the up-regulated secretion of the immunosuppressive cytokine, TGFβ. Secretion of the Th1 type (IL-2 and INFγ) cytokines was diminished in mice treated with pCDR1 for the prevention of disease induction. In mice treated with pCDR1, when clinical symptoms were already established, Th1- as well as the Th2-type cytokines were immunomodulated to levels similar to those detected in healthy mice.

In the present study, the beneficial effects of pCDR1 were demonstrated in a model of experimental SLE. That pCDR1 injected only weekly five times in PBS during the immunization period with the 16/6Id for disease induction prevented disease development is of great significance, taking into consideration that the human anti-DNA 16/6Id used for disease induction is a multideterminant molecule. Indeed, the latter treatment led to beneficial effects on all measured clinical manifestations. It is noteworthy that the effect of pCDR1 was long-lasting because the mice were treated at the time of disease induction, and the beneficial effects were still observed at sacrifice (about 7–8 months after treatment had stopped).

Treating an already existing experimental SLE with pCDR1 is relevant for application to human disease, because in the latter case, treatment can start after patients are diagnosed as afflicted with SLE. Note that a relatively brief treatment regimen (10 weekly injections of 100 μg/mouse of the CDR1-based peptide) ameliorated all tested clinical manifestations of the complex systemic disease. The benefits of treatment with pCDR1 lasted for at least 2 months (mice were then killed) without further treatment. The CDR1-based peptide was also capable of preventing the lupus-like disease of (NZBxNZW)F1 mice (12) and, furthermore, it could down-regulate the clinical symptoms of an already developed disease in the latter SLE-prone mice and in MRL/lpr/lpr mice that also develop spontaneously SLE (H.Z., E.E., A. Meshorer, and E.M., unpublished work). The efficacy of the peptide based on the CDR1 of the murine 5G12 mAb in affecting the disease of (NZBxNZW)F1 mice is probably because of the high similarity between 5G12 mAB and anti-DNA Abs isolated from the SLE-prone mice (13, 14). Auto-Ab-derived peptides were recently shown to either delay disease onset, prolong survival, or ameliorate disease manifestations in (NZBxNZW)F1 mice (15–18).

It should be noted that both prevention and treatment with pCDR1 did not abolish completely the production of DNA-specific Abs. Nevertheless, a significant amelioration was determined in all of the clinical manifestations that were tested. These results are in agreement with our previous publications, in which experimental SLE was treated with methotrexate (19), tamoxifen (20), or methimazole (21). Beneficial effects of treatment of SLE-prone mice without complete depletion of measurable auto-Abs were reported by others as well (22–24).

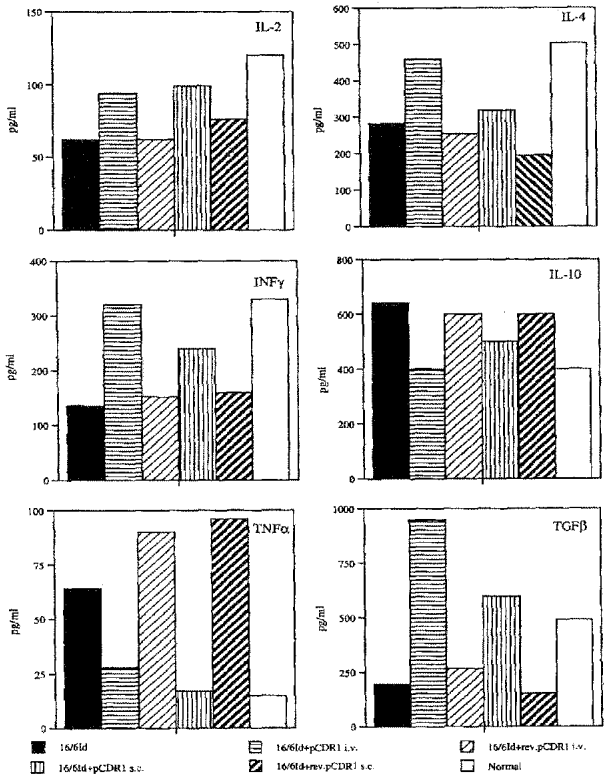

Fig. 5. The effect of treatment with the CDR1-based peptide on the cytokine pattern. BALB/c mice (20 mice/group) were immunized with 16/6 Id and 3.5 months later injected i.v. or s.c. with pCDR1. Two mice were killed monthly, and their spleen cells were stimulated with 16/6 Id. Supernatants were analyzed for cytokine secretion. Results are representatives of two experiments.

Cytokines have been suggested to play an important role in immune dysregulation observed in lupus-prone mice and in patients with SLE (25, 26). We have previously shown that the development of experimental SLE in mice involves two stages: first, increased production of Th1-type (IL-2, INFγ) followed by a significant increase in the secretion of Th2-type (IL-4, IL-10) cytokines (associated with decreased levels of both IL-2 and INFγ). Approximately 7 months after disease induction, when mice exhibit the full-blown disease, secretion of IL-2, INFγ, and IL-4 is diminished. High levels of the proinflammatory cytokines, TNFα and IL-1, are detected and maintained throughout disease course (11). A shift from Th1- to Th2-type cytokines has been reported in SLE patients (27), and it has been shown that both Th1- and Th2-type cells are down-regulated with disease progression in the patients (28).

A decrease in IL-2 and INFγ has been observed in mice treated with pCDR1 for prevention of SLE induction. The decline in the Th1-type cytokines was for a relative short period (about 2 months); nevertheless, it covered the period in which a

Th1 environment has been shown to be essential for induction of experimental SLE (11). INFγ plays a major role in the pathogenesis of SLE. Administration of INFγ along with disease induction aggravated disease manifestations (29). Further, MRL/lpr/lpr mice deficient of INFγ gene (30) or the INFγ receptor gene (31) were protected from disease development, as were (NZBxNZW)F1 mice treated with anti-INFγ Abs (32) or INFγ-soluble receptors (33). In addition to down-regulation of Th1-type cytokines, a reduction in TNFα and an increase in TGFβ were observed. Thus, a short course of pCDR1 administration resulted in a reduced production of the pathogenic cytokine TNFα, with a diminished production of IL-2 and INFγ and an increased secretion of TGFβ. The latter shifts in cytokine pattern resulted in the inhibition of disease development.

The beneficial effects of treating with pCDR1 mice with an established disease were associated with a significant decrease in the secretion of TNFα. This cytokine was reported to accelerate the kidney disease when injected to different experimental model animals (34); increased TNFα mRNA was observed in renal, splenic, and lung tissues of SLE-prone mice (25, 26), and high levels of soluble TNFα receptor were found in the sera of active SLE patients (35). Treatment of SLE-afflicted mice with either methotrexate or tamoxifen resulted in beneficial effects that were associated with a diminished secretion of TNFα (19, 20). Further, mice with SLE benefited significantly from treatment with either anti-TNFα or pentoxiphylline that was shown to reduce the levels of TNFα (36). It is very likely that pCDR1 modulates SLE manifestations by down-regulating TNFα production, which results also in restoration of the profile of Th1 and Th2 cytokines to levels similar to those observed in healthy mice (Fig. 5).

Treatment with the CDR1-based peptide resulted in a significant increase in the secretion of the immunosuppressive cytokine, TGFβ. Elevated levels of TGFβ were detected in mice that were treated with pCDR1 either for prevention or for curing an established disease. TGFβ-null mice were shown to develop autoimmune manifestations that resemble SLE (37), and the injection of a TGFβ cDNA expression vector into the skeletal muscle of the lupus-prone MRL/lpr mice decreased auto-Ab production (38). Both constitutive and stimulated levels of TGFβ are lower in patients with SLE, and the high IgG production seen in patients with SLE is attributed in part to low levels of TGFβ (39). It is not clear yet whether the elevated levels of TGFβ down-regulate the pathogenic cytokine TNFα or whether the administration of pCDR1 results in down-regulation of TNFα concomitant with an up-regulation of TGFβ. Nevertheless, the apparent effect of the immunomodulation of the above cytokines is a significant amelioration of the clinical manifestations of experimental SLE.

Treatment of SLE to date is not specific. The corticosteroids and immunosuppressive agents used to treat patients affect the function of the immune system and could be accompanied with severe adverse effects. The CDR1-based peptide, on the other hand, was shown to immunomodulate specifically experimental SLE that was induced by the pathogenic auto-Ab. It was also shown by us to affect beneficially the SLE-like disease that develops spontaneously in (NZBxNZW)F1 and in MRL/lpr/lpr mice. On the basis of its efficacy in the different models of SLE, pCDR1 might be considered a candidate for therapy of human SLE.

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1. Mendlovic, S., Brocke, S., Shoenfeld, Y., Ben-Bassat, M., Meshorer, A., Bakimer, R. & Mozes, E. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2260–2264.
2. Mendlovic, S., Brocke, S., Fricke, H., Shoenfeld, Y., Bakimer, R. & Mozes, E. (1990) *Immunology* **69,** 228–236.
3. Mendlovic, S., Fricke, H., Shoenfeld, Y. & Mozes, E. (1989) *Eur. J. Immunol.* **19,** 729–734.
4. Waisman, A., Mendlovic, S., Ruiz, J. P., Zinger, H., Meshorer, A. & Mozes, E. (1993) *Int. Immunol.* **5,** 1293–1300.
5. Shoenfeld, Y., Ben-Bassat, M. & Mozes, E. (1991) *Immunology* **73,** 421–427.
6. Waisman, A. & Mozes, E. (1993) *Eur. J. Immunol.* **23,** 1566–1573.
7. Waisman, A., Ruiz, P. J., Israeli, E., Eilat, E., Konen-Waisman, S., Zinger, H., Dayan, M. & Mozes. E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4620–4625.
8. Schnolzer, M., Alewood, P. F. & Kent, S. B. H. (1992) *Int. J. Pept. Protein Res.* **40,** 180–193.
9. Shoenfeld, Y., Hsu-Lin, S. C., Gabriels, J. E., Silberstein, L. E., Furie, B. C., Furie, B., Stollar, B. D. & Schwartz, R. S. (1982) *J. Clin. Invest.* **70,** 205–208.
10. Waisman, A., Shoenfeld, Y., Blank, M., Ruiz, P. J. & Mozes, E. (1995) *Int. Immunol.* **7,** 689–696.
11. Segal, R., Bermas, B. L., Dayan, M., Kalush, F., Shearer, G. M. & Mozes, E. (1997) *J. Immunol.* **158,** 3009–3016.
12. Eilat, E., Zinger, H., Nyska, A. & Mozes, E. (2000) *J. Clin. Immunol.* **20,** 268–278.
13. Wloch, M. K., Allexander, A. L., Pippen, A. M. N., Pisetsky, D. S. & Gilkson, G. S. (1997) *J. Immunol.* **158,** 4500–4506.
14. Tillman, D. M., Jou, N. T., Hill, R. J. & Marion, T. N. (1992) *J. Exp. Med.* **176,** 361–379.
15. Gaynor, B., Putterman, C., Valadon, P., Spatz, L., Scharff, M. & Diamond, B. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1955–1960.
16. Singh, R. R., Ebling, F. M., Sercarz, E. E. & Hahn, B. H. (1995) *J. Clin. Invest.* **96,** 2990–2996.
17. Jouanne, C., Avrameas, S. & Payelle-Brogard, B. (1999) *Immunology* **96,** 333–339.
18. Kaliyaperumal, A., Michaels, M. A. & Datta, S. K. (1999) *J. Immunol.* **162,** 5775–5783.
19. Segal, R., Dayan, M., Zinger, H. & Mozes, E. (1995) *Clin. Exp. Immunol.* **101,** 66–72.
20. Dayan, M., Zinger, H., Kalush, F., Mor, G., Zaltzman, Y., Kohen, F., Sthoeger, Z. & Mozes, E. (1997) *Immunology* **90,** 101–108.
21. Singer, D. S., Kohn, L. D., Zinger, H. & Mozes, E. (1994) *J. Immunol.* **153,** 873–880.
22. Nicoletti, F., Zaccone, P., Magro, G., Barcellini, W., Cavallaro, V., Belli, G. Cocuzza, C., di Marco, R. & Meroni, P. L. (1994) *Scand. J. Immunol.* **40,** 549–556.
23. Erausquin, C., Merino, R., Izui, S., Fernandez-Sueiro, L., Saez, F., Fernandez, F., Rodriguez-Valverde, V. & Merino, J. (1995) *Cell. Immunol.* **161,** 207–212.
24. Macanovic, M., Sinicropi, D., Shak, S., Baughman, S., Thiru, S. & Lechmann, P. J. (1996) *Clin. Exp. Immunol.* **106,** 243–252.
25. Handwerger, B. S., Rus, V., da Silva, L. & Via, C. S. (1994) *Springer Semin. Immunopathol.* **16,** 153–180.
26. Horwitz, D. A. & Jacob, C. O. (1994) *Springer Semin. Immunopathol.* **16,** 181–200.
27. Hagiwara, E., Gourley, M. F., Lee, S. & Klinman, D. M. (1996) *Arthritis Rheum.* **39,** 379–385.
28. Bermas, B. L., Petri, M., Goldman, D., Mittleman, B., Miller, M. W., Stocks, N. I., Via, C. S. & Shearer, G. (1994) *J. Clin. Immunol.* **14,** 169–177.
29. Amital, H., Levi, Y., Blank, M., Langevits, P., Afek, A., Nicolleti, F., Kupolovic, J., Gilburd, B., Meroni, P. L. & Shoenfeld, Y. (1998) *Lupus* **7,** 445–454.
30. Haas, C., Ryffel, B. & Le Hir, M. (1997) *J. Immunol.* **158,** 5484–5491.
31. Peng, S. L., Moslehi, J. & Craft, J. (1997) *J. Clin. Invest.* **99,** 1936–1946.
32. Jacob, C. O., Van Der Meide, P. H. & McDevitt, H. O. (1987) *J. Exp. Med.* **166,** 798–803.
33. Ozmen, L., Roman, D., Fountoulakis, M., Schmid, G., Ryffel, B. & Garotta. G. (1995) *Eur. J. Immunol.* **25,** 6–12.
34. Tomosugi, N. I., Cashman, H. J., Hay, H., Pusey, C. D., Evans, D. J., Shaw, A. & Rees, A. J. (1989) J. *Immunol.* **142,** 3083–3090.
35. Aderka, D., Wysenbec, A., Engelmann, H., Cope, A. P., Brennan, F., Molad, Y., Hornik, V., Levo, Y., Maini, R. N., Feldmann, M. & Wallach, D. (1993) *Arthritis Rheum.* **36,** 1111–1120.
36. Segal, R., Dayan, M., Zinger, H. & Mozes, E. (2000) *Lupus,* in press.
37. Yaswen, L., Kulkarni, A. B., Fredrickson, T., Mittleman, B., Schiffmann, R., Payne, S., Longenecker, G., Mozes, E. & Karlsson, S. (1996) *Blood* **87,** 1439–1445.
38. Raz, E., Watanabe, A., Baird, S. M., Eisenberg, R. A., Parr, T. B., Lotz, M., Kipps, T. J. & Carson, D. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4523–4527.
39. Ohtsuka, K., Gray, J. D., Stimmler, M. M., Toro, B. & Horwitz, D. A. (1998) *J. Immunol.* **160,** 2539–2545.

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Modulation of auto reactive responses of peripheral blood lymphocytes of patients with systemic lupus erythematosus by peptides based on human and murine anti-DNA autoantibodies

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SUMMARY

Two peptides, based on the sequences of the complementarity-determining regions (CDR) 1 and 3 of a pathogenic murine monoclonal anti-DNA autoatibody that bears the 16/6 idiotype (Id), were shown to either prevent or treat an already established systemic lupus erythematosus (SLE) in two murine models of lupus. Two additional peptides based on the human monoclonal anti-DNA, 16/6 Id were synthesized. This study was undertaken in order to investigate the ability of the CDR-based peptides to immunomodulate SLE-associated responses of peripheral blood lymphocytes (PBL) of SLE patients. PBL of 24 of the 62 SLE patients tested proliferated *in vitro* following stimulation with the human 16/6 Id. Peptides based on the CDRs of both the human and murine anti-DNA autoantibodies inhibited efficiently and specifically the 16/6 Id-induced proliferation and IL-2 production. The latter inhibitions correlated with an up-regulated production (by 2'5-3'5-fold) of the immunosuppressive cytokine, TGF-{3 Overall, the results of our study demonstrate that the CDR-based peptides are capable of down-regulating *in vitro* autoreactive T cell responses of PBL of SLE patients, Thus, these peptides are potential candidates for a novel specific treatment of SLE patients.

Keywords CDR-based peptides IL-2 immunomodulation systemic lupus erythematosus TGF-{3

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of a variety of autoantibodies, impairment of Band T cell functions, cytokines production and immune complex deposition accompanied by systemic clinical mamfestatIOns (e.g. neurological, dermal, haematologlcal, musculoskeletal and renal) [1]. The exact pathogenesis of the disease, as well as the autoantIgen(s) in SLE, are not yet defined, The common idiotype designated 16/6 Id was detected on anti-DNA antibodies of about 50% of SLE patients [2,3], and its presence was shown to correlate with disease activity [4]. Moreover, peripheral blood lymphocytes (PBL) obtained from SLE patients responded specifically to 16/6 Id stimulation [3-5]. Previous studies from our laboratory demonstrated the induction of experimental SLE in naive (not SLE-prone) mice by immunization with the human 16/6 Id monoclonal antibody (MoAb) or with the murine anti-DNA 16/6 Id, 5G12 MoAb [6,7]. The immunized mice developed high levels of autoantibodies, including antibodies bearing the 16/6 Id, as well as SLE-related clinical manifestations (e.g. leukopenia, thrombocytopenia and renal Inflammation) [6-8]. Autoantibodies Isolated from the diseased mice including the 5G12 MoAb were shown to be highly homologous to anti-DNA MoAb Isolated from the SLE-prone (NZB x NZW)F1 mice [9], supporting further the Importance of the 16/6 Id in SLE.

Two peptides, based on the sequences of the complementarity-determining regions (CDR) of the pathogenic murine anti-DNA 16/6 Id (5G12), namely mCDR1 and mCDR3, were designed and synthesized [10]. Those peptides were shown to be Immunodominant T-cell epitopes in non-autoImmune (e.g. BALB/c) and lupus-prone (NZB x NZW) F1 mice [10-12]. Furthermore, treatment With these peptides ameliorated the SLE-like clinical manifestations and decreased autoantibody production of both, spontaneous (NZB x NZW) F1 and 16/6 Id-induced SLE [10,12-15]. Experiments performed with single amino acid substituted analogues for mCDR1 (39 analogues) and

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for mCDR3 (17 analogues) as well as with truncated peptides (either at the N and/or at the C-terminus), indicated that the original mCDRI and mCDR3 were the best immunomodulators of SLE associated responses [15,16].

To investigate further the role of the CDR-based peptides in the treatment of human SLE we have synthesized two additional peptides, based on the sequences of CDRs of the human anti-DNA Hi/6 Id, namely hCDRI and hCDR3. In the present study, we have examined the ability of the murine and the newly synthesized human CDR-based peptides to inhibit the 16/6 Id specrnc stimulation (proliferation) of PBL obtained from patients with SLE. We demonstrate here that the peptides inhibited specifically the 16/6 Id-induced proliferation. This inhibition correlated with a reduction in IL-2 secretion and with an up-regulated production of the immunosuppressive cytokine, TGF-/l

PATIENTS MATERIALS AND METHODS

Patients

Sixty-two patients, nine males (14·5%) and 53 females (85·5%), with SLE participated in our study. The mean age at diagnosis was 32·95 ± 12·92 (range 12-61) years and the mean follow-up period was 10·98 ± 10·76 (range 1-32) years. All patients fulfilled at least four of the American College of Rheumatology (ACR) revised diagnostic criteria for SLE [17]. Patients were recruited from three Israeli Medical Centers (Kaplan, Rehovot; Ichilov, Tel Aviv; Asaf-Harofeh, Rishon Lezion). Disease activity was determined according to the SLEDAI lupus activity index [18]. A control group of 36 sex- and age-matched healthy control volunteers was studied concomitantly with the SLE patients. All participants signed an informed consent form prior to the initiation of the study. The study was approved by the Ethical Committee of the Medical Center.

Monoclonal antibody

The human anti-DNA MoAb that bears the 16/6 Id (IgGl/k) has been characterized previously [19], The MoAb waJl secreted by hybridoma cells that were grown in culture and were purified by using a protein G-sepharose column (Pharmacia, Fine Chemicals, Uppsala, Sweden) [14].

Synthetic peptides

Synthetic peptides based on the CDRI and CDR3 of the murine monoclonal anti-DNA 16/6 Id and of the human anti-DNA 16/6 Id MoAbs [7,19] were prepared as described previously [20]. The amino acid sequences of the human and murine CDR-based peptides are presented in Table 1. As shown in the Table 1, the peptides based on the CDR of the human and murine anti-DNA antibodies share a significant number of amino acids at identical positions. The reversed peptides that were synthesized in the reversed order of mCDRI and mCDR3 (revmCDRI; revmCDR3) were used as control.

Proliferative responses

PBL were isolated from heparinized venous blood by FicolJ-Hypaque (Pharmacia) density-gradient centrifugation [21]. All assays were performed in triplicate in flat-bottomed microtitre plates (Falcon, Becton Dickinson, Oxmard, CA, USA) in whicn 2×10^5 PBL were cultured in enriched RPMI-1640 as described [3]. The PBL were exposed to various concentrations (0,1-40 J.Lg/well) of thc human anti-DNA 16/6 Id MoAb with and without the addition of the various CDR-based peptides. Phytohaemagglutinin (PHA; 2 J.Lg/wcll)was used as a control for culture conditions at each experiment. The cultures were incubated in 7·5% C02 at 37°C for 6 days. Eighteen hours before the cells were harvested, PH]thymidine (0·5 .ll Ci of 5 Ci/mmol) (Nuclear Research Center, Negev, Israel) was added to all cultures. Results are expressed as the mean thymidine incorporation in counts per minute (cpm) of triplicate culture ± s.d., or as stimulation index (SI; the ratio of mean cpm at the optimal concentration of the human 16/6 Id to the mean cpm in the presence of medium alone). SI ~ 2 was considered a positive response [3]. Inhibition (the ratio of mean cpm in the presence of the 16/6 Id and various CDR-based peptides to the mean cpm with the 16/6 Id without the CDR-based peptide) above 50% was considered positive.

Assessment of cytokine production

Supernatants were collected 48 h following the initiation of the cultures and stored at -70°C. IL-2 was determined by using the IL-2-dependent CTLL linc as described previously [22]. *TGF-,B* was determined by an enzyme-linked immunosorbant assay (ELISA). Briefly, plates were coated with the recombinant human TGF-,81 sRIIIFc chimera (R&D Systems Inc., Minneapolis, MN, USA). For detection wc used biotin-labelled anti-human *TGF-,B* antibody (R&D Systems Inc.) [14]. In a separate set of experiments, PBL (2 x 105) of thc patients were incubated with the CDR-based peptides in the absence of the human anti-DNA 16/61d MoAb. Supernatants were collected after 48 h and tested for *TGF-,B* secretion as above.

Statistical analysis

Results presented as mean ± s.d. Chi-square, Wilcoxon and *t-tests* were employed for statistical analysis. *P:::;* 0·05 was considered significant.

Table 1. Amino acid sequences of peptides based on the CDRs of murine and human monoclonal anti-DNA 16/61d autoantibodies

mCDRI	T	Q	Y	Y	M	Q	Y	V	K	Q	S	~	E	K	S	L	~	Y	I	Q		
hCDRI		Q	Y	Y	W	S	Y	IRQ	R	Q	P	~	G	K	G	E	~	Y	I	Q		
mCDR3	Y	Y	~	A	R	F	I	W	E	PYA	Y	A	M	Q	Y	W	G	Q	Q	S		
hCDR3	Y	Y	~	A	R	G	I	L	R	G	G	W	N	Q	V	D	Y	Y	Q	M	D	V
Reversed mCDRI	G	I	W	E	L	S	K	E	P	S	Q	K	V	W	Q	M	Y	Y	G	T		
Reversed mCDR3	S	G	Q	G	W	Y	D	MAY A		Y	PEW E		W	L	F	R	A	C	Y	Y		

Peptides based on the CDRI and CDR3 of the murine autoantibody were designated mCDRI and mCDR3, respectively, and peptides based on the CDRs of the human autoantibody were designated hCDRI and hCDR3.

RESULTS

Proliferative capacity and clinical characterization of SLE patients

It was of interest to find out whether the peptides, based on CDR1 and CDR3 of monoclonal anti-DNA 16/6 Id antibodies, are capable of inhibiting the specific proliferative responses of PBL of SLE patients to the human 16/6 Id. Furthermore, we wanted to compare the inhibitory capacity of the peptides based on the CDRs of the murine anti-DNA autoantibody to that of the newly synthesized peptides based on CDR1 and CDR3 of the human monoclonal anti-DNA 16/6 Id (Table 1). To this end, we first had to identify the patients whose PBL could be stimulated to proliferate by the human 16/6 Id. Therefore, PBL of 62 consecutive SLE patients were cultured in the presence of the human 16/6 Id and their proliferative responses and ability to secrete IL-2 were determined. PBL of 24 of the total of 62 (39%) and of 23 of 55 (42%) SLE patients tested responded (SI = 2, range 2-5·6) by proliferation and by IL-2 secretion .(SI = 2, range 2-60), repectively. The frequency of responders III the group of SLE patients was lower than that observed in the group of healthy donors that was tested as control. Thus, PBL of 21 of a total of 36 (58%) healthy donors responded by proliferation to the 16/6 Id. These results are similar to those obtained in our previous studies [3,4]. The extent of proliferation (SI levels) was similar for the SLE patients and for the healthy controls who responded to the 16/6 Id. However, the optimal response to the 16/6 Id of PBL of the control donors was observd at higer concntrations .of16/6 I? as compared to the SLE patlents (FIg. 1). It IS shown III the FIg. 1 that whereas PBL of most SLE patients responded to the 16/6Id at a concentration of 1-10 ,uglwell, PBL of healthy controls responded mainly to concentrations of 5-20 ,uglwell (P = 0·016 for the 20 ,uglwell dose when numbers of responders out of the healthy donors and SLE patients were compared).

No differences could be demonstrated between gender and age of SLE patients that responded to the 16/6 Id and of the non-responder group of patients. However, the patients whose PBL proliferated in response to the 16/6 Id were sick for a shorter period of time (a mean of 9.78 ± 8.36 *versus* 11.73 ± 12.06 years for responders and non-responders, respectively; P = 0.036). Table 2 summarizes the clinical characterization of the 16/6 Id-specific responder and non-responder groups of SLE patients. As can be seen in the Table 2, both groups were similar in most SLE-related clinical manifestations. The disease activity score (SLE-DAI) and the number of SLE diagnostic criteria were also similar in the two groups. Nevertheless, a higher frequency of neurological (both siezures and psychosis) and haematological involvement and a lower rate of renal involvement were noted in the responder group of patients in comparison to the group of non-responders. However, probably because of the low number of patients in the relevant subgroups, the above differences did not reach statistical significance. Moreover, relatively less responder patients were determined between those treated with either steroids or cytotoxic agents at the time of the study. It is noteworthy that significantly more patients who never received steroids responded to the 16/6 Id in comparison to the non responder group (54% *versus* 21%; P = 0.023).

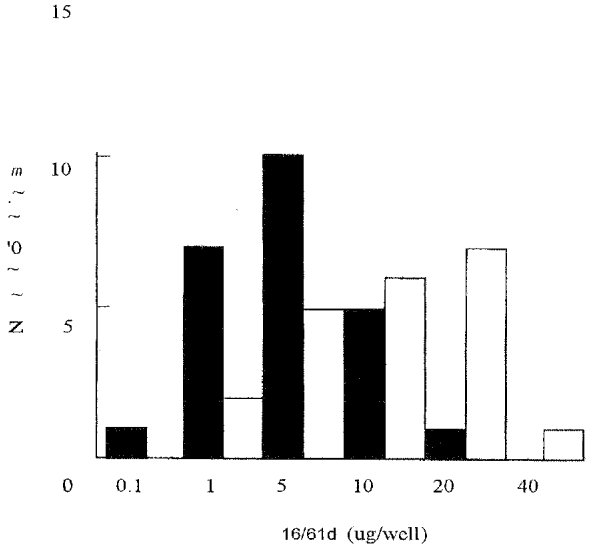

Fig. 1. Concentrations of 16/6 Id required for optim1 stunulatI?n of BL of SLE patients and of healthy controls. PBL were stImulated WIthvanous concentrations (0·1-40 JIg/well)of the 16/6 Id. The concentratIon Yldmg the highest stimulation index was defined as optimal for triggering a proliferative response. _, SLE patients; D, healthy controls.

In vitro *inhibition of* 16/6 *Id-induced stimulation of PBL of SLE patients*

The ability of the peptides, based on the CDRs of the munne (mCDR1 and mCDR3) and of the human (hCDR1 and hCDR3) autoantibodies, to inhibit the proliferative responses of PBL of SLE patients and of healthy controls to the human 16/6 Id was tested. Table 3 summarizes the inhibitory capacity of the peptides. Peptides mCDR1, hCDR1 and hCDR3 inhibited the proliferative response to the 16/6 Id of PBL of a similar number of SLE patients (15/19, 16/19 and 15/19, respectively). Peptide mCDR3 inhibited the proliferation of PBL of fewer patients (6/19) when added to cultures of PBL stimulated with the 16/6 Id. The mean maximum percentage inhibition was comparable for all four CDR based peptides tested (Table 3). When the ability of the peptides to inhibit the IL-2 secretion of PBL stimulated by the human 16/6 Id was tested mCDR1 and hCDR1 inhibited the secretion by PBL of 23/23 nd 21/23, respectively. Similarly, secretion of IL-2 by PBL of 19/23 tested individuals was inhibited by either mCDR3 or hCDR3 (Table 3b). Inhibition of proliferative responses correlated directly with IL-2 inhibition by the CDR-based peptides. Thus, inhibition of IL-2 secretion was observed in all cases where inhibition of proliferation were determined. It is noteworthy that the efficacy of the CDR-based peptides to inhibit the proliferative responses of PBL of healthy donors to the 16/6 Id was much lower than that observed for PBL of SLE patients. The proliferation of PBL of only four, 10, five and nine of the 18 healthy controls tested was inhibited by mCDR1, hCDR1, mCDR3 and hCDR3, respectively. (Table 3c). The. effect of the CDR-based peptides on IL-2 secretIOn by 16/6 Id-stimulated PBL of healthy controls was not tested .

The inhibition of responses of PBL to the human 16/6 Id was shown to be specific, because two control peptides that were synthesize d III the reverse d order of mCDR1 and mCDR3 (a 1: ble1 could not inhibit the 1/6 Id-spel c pr 1erative responses. Figure 2 represents a typical expenment with PBL of one SLE

Table 2. Clinical and laboratory characterization of SLE patients

	All patients	Responders	Non-responders
(a) Diagnostic criteria			
No. of patients (%)	62 (100)	24 (39)	38 (61)
Malar rash	19/62 (30·1)	8/24 (33·3)	11/38 (29)
Discoid rash	9/62 (15)	3/24 (12·5)	6/38 (16)
Photosensitivity	21/62 (34)	9/24 (37·5)	12/38 (32)
Mucosal ulcers	17/62 (27·4)	8/24 (33·3)	9/38 (23·7)
Arthritis	46/62 (74'2)	19/24 (79'2)	27/38 (71)
Serositis	14/62 (22·6)	5/24 (20·8)	9/38 (23·7)
Neurological disorders:f:	5/62 (8'1)	4/24 (16·7)	1/38 (2·7)
Renal disorder:f:	24/62 (38·8)	7/24 (29'2)	17/38 (44·8)
Haematological disorders:f:	44/62 (71)	19/24 (79'2)	25/38 (65·8)
ANA	61/62 (98·4)	24/24 (100)	37/38 (92,1)
a-dsDNA	54/62 (87·1)	19/24 (79'2)	35/38 (92'1)
APLA	35/62 (56·5)	12/24 (50·0)	23/38 (60·53)
(b) Disease activity			
SLEDAI score	6·65 ± 5·12	7·29±1·06	6·24 ± 0·84
Number of ACR diagnostic criteria	5·44 ± 1·39	5·54 ± 0·33	5,34 ± 0·2
(c) Current treatmentt			
NSAIDS	17/62 (27·4)	6/24 (25)	11/38 (29)
Anti-malarial	37/62 (59·7)	15/24 (62·5)	22/38 (57·9)
Steroids:f:	33/62 (53,2)	11/24 (45·8)	22/38 (57·9)
Cytotoxic:f:	10/62 (16'1)	2/24 (8·3)	8/38 (21)

'Clinical involvement was defined according to the ACR revised criteria [16]. Anti-nuclear antibodies (ANA) and anti-dsDNA antibodies were determined by Hep2 cells and *Crithidia luciliae*, respectively. Anti-phospholipid antibodies (APLA) were defined as reactivity in one or more of the following assays: false positive VDRL, lupus anticoagulant (LAC) or ELISA for anticardiolipin antibodies.

tThe antimalarial agent, hydroxycWoroquine, was used at a dose of 200-400 mg/day; steroid treatment was defined as a daily dose ~ 5 mg of prednisone; cytotoxic agents used were cyclophosphamide *(0·75-1·0 g/m²;* monthly) or azathioprine (100-150 mg/day). :f:Parameters for which tendency was observed towards differences between the two groups of responder and non responder SLE patients.

patient. It can be seen that, whereas all four peptides based on CDR1 and CDR3 of the murine and human anti-DNA 16/6 Id inhibited efficiently the proliferative response to the 16/6 Id in this SLE patient, neither of the control reversed peptides could do so. The specificity of the inhibition by the CDR-based peptides was tested further. Thus, because PBL of all patients who responded to the 16/6 Id proliferated to PHA as well, the ability of the CDR-based peptides to inhibit the latter responses was tested. Figure 3 demonstrates results of a representative experiment. Neither peptide could inhibit the proliferative responses to the mitogen, confirming further the specificity of their inhibitory effects (Fig. 3).

Up-regulation of the secretion of TGF-β by the CDR-based peptides

Because we have shown that down-regulation of SLE manifestations by the CDR-based peptides in murine models is associated with up-regulated secretion of the immunosuppressive cytokine, TGF-β [14], it was of interest to find out whether incubation of PBL in the presence of the latter CDR-based peptides will stimulate the secretion of TGF-β. To this end, supernatants of PBL of SLE patients that were incubated in culture with the 16/6 Id and with either of the CDR-based peptides, were tested for the content of TGF-β. The results are summarized in Table 4. It can be seen that in most cases the CDR-based peptides up-regulated significantly the secretion of TGF-β by the PBL. The up-regulation ofTGF-β correlated directly with the inhibition of proliferative responses and IL-2 secretion. The up-regulation of TGF-β secretion by the CDR-based peptides is specific because the reversed CDR-based peptides, used as controls, did not trigger the secretion of TGF-β to levels above those observed in the presence of the 16/6 Id alone (Fig. 4).

Thus, the peptides based on the human and murine auto antibodies are capable of inhibiting the proliferative responses and IL-2 secretion of PBL of SLE patients that are stimulated by the human 16/6 Id. The latter correlates with an increased production of the immunosuppressive cytokine, TGF-β.

It was of interest to find out whether the various CDR-based peptides are capable of up-regulating the secretion of TGF-β by PBL of the various patients when incubated in the absence of the 16/6 Id. To this end PBL of a group of patients were incubated with the CDR-based peptides. Supernatants of the cultures were tested for the content ofTGF-β. The results demonstrated that in the majority of the cases incubation of PBL with the peptides caused a moderate but significant increased secretion of 1·5-3·5-fold compared to the levels of TGF-β secreted by cells that were incubated in the presence of medium alone. Figure 5 demonstrates the results obtained with PBL of a representative SLE patient. It is noteworthy that the levels ofTGF-β secreted by PBL

Table 3. Inhibition of 16/6 Id-induced stimulation of PBL by the CDR-based peptides

Peptide	Inhibitory activity%*	Maximum inhibition%†
(a) SLE patients - proliferation		
mCDR1	79 (15/19)	64 ± 9
hCDR1	84 (16/19)	68 ± 10
mCDR3	32 (6/19)	75 ± 8
hCDR3	79 (15/19)	72 ± 8
(b) SLE patients - IL-2 secretion		
mCDR1	100 (23/23)†	88 ± 16
hCDR1	91 (21/23)	84 ± 31
mCDR3	83 19/23)	58 ± 41
hCDR3	83(19/23)	78 ± 34
(c) Healthy controls - proliferation		
mCDR1	22 (4/18)	75 ± 7
hCDR1	56 (10/18)	68 ± 10
mCDR3	28 (5/18)	58 ± 3
hCDR3	50 (9/18)	68 ± 8

*The percentage of SLE patients or healthy controls in whom the responses of the PBL were inhibited by the CDR based peptides (above 50% inhibition, as in Methods). Parentheses represent the number of patients or controls out of the groups tested whose PBL were inhibited. †The mean ± s.d. of percentage maximum inhibition by the CDR-based peptides. Proliferative responses in the presence of the 16/6 Id without any CDR-based peptide were considered as 100%. :j:IL-2secretion in the presence of 16/6 Id alone was considered as 100%. Inhibition of 50% or more was considered significant.

Table 4. Up-regulation ofTGF-f3 secretion of 16/6 Id-induced stimulation of PBL of SLE patients with CDR peptides

Peptide	Up-regulation of TGF-f3 %	Maximum up-regulation %
mCDR1	84 (16/19)	259 ± 240
hCDR1	100 (19/19)	305 ± 221
mCDR3	89 (17/19)	269 ± 170
hCDR3	100 (19/19)	338 ± 242

Secretion ofTGF-f3 in the presence of 16/6 Id alone (mean 636 ± 25 *pg*/ml) was considered as 100%. Results are expressed as percentage secretion above that in the presence of 16/61d alone.

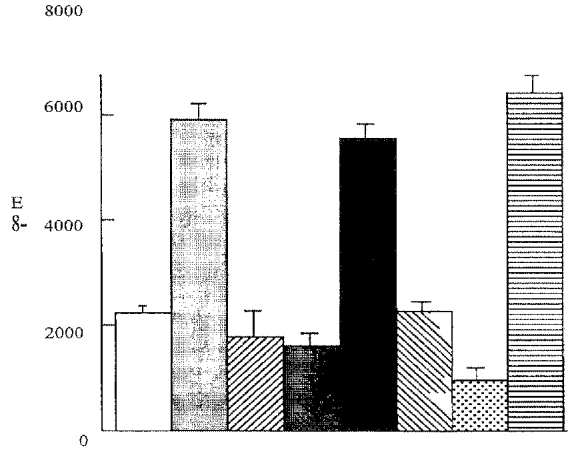

Fig. 2. Inhibition of 16/6 Id-stimulated proliferatIOn by peptIdes based on the human or munne anti-DNA autoantibodies. PBL of a representative SLE patient were cultured for 6 days in the presence of 16/6 Id with or without the various peptides based on the human (hCDR1, hCDR3) or murine (mCDR1, mCDR3) autoantibodies or with the control peptides (revmCDR1, revmCDR3). For the last 18 h 0'5p,Ci of [3H]thymidine was added. Thereafter cells were harvested and radioactivity was counted. Results are expressed as mean cpm of triplicates ± s.d. D, Medium; D, 16/61d; D, 16/61d+mCDR1; D, 16/61d+hCDR1; ., 16/61d+revmCDR1; D, 16/61d+mCDR3; D, 16/61d+hCDR3; D, 16/61d+revmCDR3.

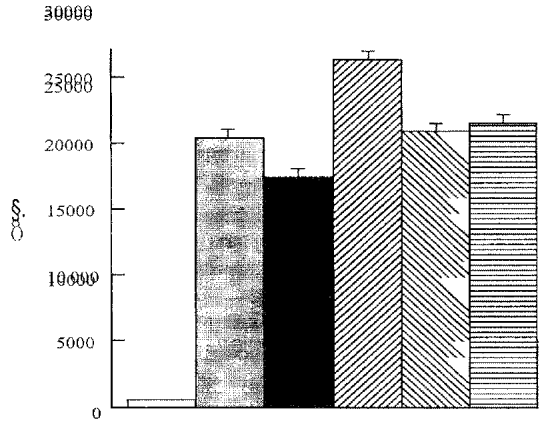

Fig. 3. The CDR-based peptides do not affect the proliferative responses to PHA. PBL of a representative SLE patient were incubated in the presence of 2 pg/well of PHA and the various CDR-based peptIdes. ResuIts are expresse d as mean cpm 0f tnp IIcates ± s.d D, MediUrn; D, PHA • PHA+ mCDRI' D PHA+hCDRI' D PHA+ mCDR3' D PHA'+ hCDR3.

Incubated with the peptide only are lower than those measured In supernatants of PBL that were In culture with the 16/6 Id MoAb and the CDR-based peptides. Nevertheless, the results demonstrate that the peptides are capable of stimulating the secretion of the immunosuppressive cytokine TGF-fJ.

DISCUSSION

The main findings of the present study are that peptides based on the CDRs of human and murine pathogenic monoclonal anti-DNA 16/6 Id bearing autoantibodies, specifically inhibited the 16/6 Id-induced proliferation and IL-2 secretion by PBL obtained from SLE patients. This inhibition correlated with up-regulated secretion of the immunosuppressive cytokine, TGF-fJ. To our best knowledge this is the first study demonstrating the potential of CDR-based peptides to down-regulate *in vitro* SLE-related responses of human autoreactive T cells.

PBL obtained from 24 of 62 SLE patients responded (SI ~ 2) to stimulation by the human anti-DNA 16/6 Id autoantibody. The rate of 16/6 Id-specific proliferative responses (39%) of PBL of SLE patients is similar to our previously reported data [3-5]. We have also reported [3-5], as shown in the present study, that the

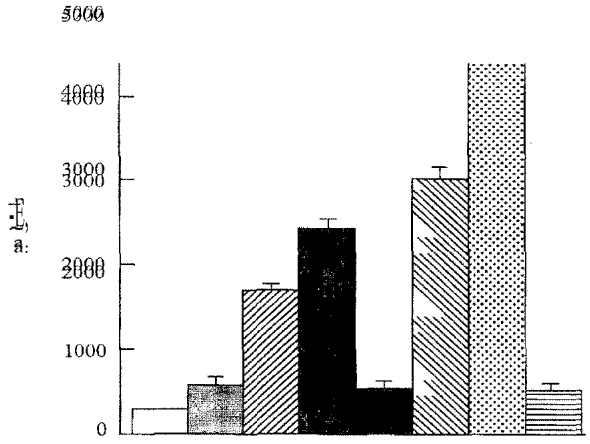

Fig. 4. Up-regulated secretion of TGF-,Btriggered by CDR-based peptides. PBL of a representative SLE patient were cultured with *16/6* Id with or without the various CDR-based peptides and the control peptides. After 48 h of incubation supernatants were collected and tested by EIS~ for the content of TGF-,B Results are expressed as mean (pg/ml) oftnphcates ± s.d. D, Medium; D, *16/6Id;* D, *16/6Id* + mCDR1; _, *16/6Id* + hCDR1; _, *16/6Id* + revmCDR1; D, *16/6Id* + mCDR3; D, *16/6Id* + hCDR3; D, *16/6Id* + revmCDR3.

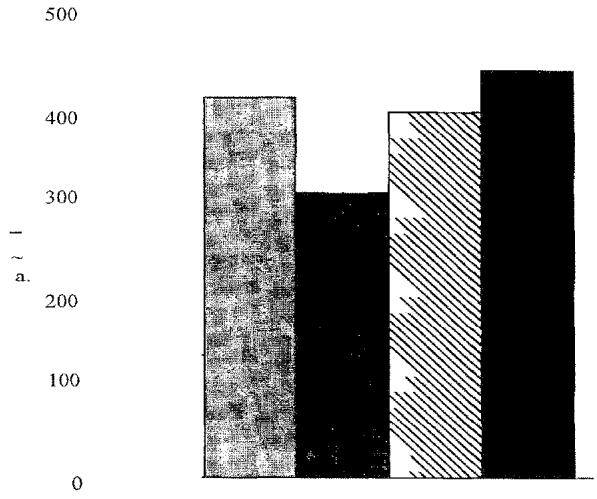

Fig. 5. The CDR-based peptides tngger PBL of SLE patients to secrete elevated levels of TGF-,B. PBL (2 x *10⁵/well)* of a representative SLE patient were cultured with 25,ug/well (total volume 200,ulIwell) of the various CDR-based peptides. After 48 h of incubation supernatants were collected and tested by ELISA for the content of TGF-,B. Results are expressed as pg/ml of secreted TGF-,B. D, Medium; D, mCDR1; _, mCDR3; ISJ, hCDR1; -, hCDR3.

proliferative rate of PBL of healthy controls to stimulation by the 16/6 Id was higher (58%) than that of SLE patients. Low rates of proliferative responses to autoantibody variable region peptides in SLE patients were also observed by Williams *et al.* [23]. The decreased capacity of PBL of SLE patients to proliferate following *in vitro* stimulation with 16/6Id may be due in part to an *in vivo* excessive spontaneous response to the 16/6 Id-related network that leads to the exhaustion of the immune cells. Other mechanisms that may account for the low T cell-responsive rate in SLE patients are probably related to the dysbalance between Th1 and Th2 cell types observed in SLE during the disease course [24-26]. Analysis of the clinical characterization of the group of SLE patients (Table 2) suggest that renal involvement and immunosuppressive treatment may also contribute to the reduced ability of PBL of SLE patients to respond *in vitro* to the 16/6 Id.

Although PBL of less SLE patients than those of healthy controls responded to *in vitro* stimulation by the 16/6 Id it is likely that the former responses were more specific and of higher affinity, as the concentrations of 16/6 Id that triggered optimal proliferative responses were lower for most PBL of most SLE patients in comparison to those required for PBL of healthy controls (Fig. 1). Alternatively, the response to lower 16/6Id concentrations may be due to the hyperresponsiveness of SLE T cells to antigenic stimulation, as was shown recently by Vratsanos *et al.* [27].

Studies from our laboratory demonstrated significant beneficial effects of treatment with mCDR1 and mCDR3 in induced [10,14,15] as well as in spontaneous (NZB x NZW) F, [13] murine SLE. It was of interest, therefore, to investigate the ability of the peptides and of the newly synthesized peptides based on the CDRs of the human 16/6 Id (Table 1) to down-regulate the 16/6 Id-specific autoreactive responses of PBL of SLE patients. The human CDR-based peptides (hCDR1, hCDR3) inhibited efficiently the 16/6 Id-induced proliferation and IL-2 secretion of PBL of patients. Peptide mCDR1 demonstrated a similar inhibitory effect, whereas mCDR3 was less effective in its inhibitory capacity (Table 3a,b). Those inhibitions were specific because two control peptides, namely reversed mCDR1 and reversed mCDR3, had no inhibitory effects on the 16/6 Id-specific proliferative responses (Fig. 2) and there were no effects of any ofthe mouse or human CDR based peptides on PHA-stimulated PBL of SLE patients (Fig. 3). The modulating effects of the various CDR-based peptides on cytokine production (IL-2 and TGF-,8) by the PBL of SLE patients is more prominent than their inhibitory effects on proliferation (Tables 3 and 4), reflecting the higher sensitivity of cytokine secretion in comparison to the proliferative responses.

In contrast to the high rate of inhibition observed for 16/6 Id-stimulated PBL of SLE patients, the effects of the various CDR-based peptides on PBL of healthy donors was less prominent (Table 3c). It is unlikely that the latter is due to insufficient amount of inhibitorory peptides because in all cultures (using PBL either of SLE patients or of healthy controls) the concentration of the CDR-based peptides was at least 10-fold higher than that of the 16/6 Id. In addition, for cases where optimal proliferations were observed with the same concentrations of the 16/6 Id, a much better inhibition was determined for stimulated PBL of SLE patients. Our inhibition experiments suggest that PBL of SLE patients and of healthy controls might recognize different determinants within the anti-DNA 16/6 Id autoantibody. Thus, T-cells of SLE patients recognize and react mainly to the CDR1 and CDR3 epitopes whereas T cells of healthy donors react probably to a variety of epitopes within the autoantibody macromolecule and therefore the inhibition of proliferation with the CDR-based peptides is less efficient in the controls.

The inhibitory effects of the CDR-based peptides, on 16/6 Id-stimulated PBL of SLE patients correlated with up-regulated

secretion of TGF-{3 (Table 4, Fig. 3). The latter imrnunosuppressive cytokine was shown to inhibit IL-1 and IL-6 production [28] and to suppress IgG production [29]. Both constitutive and stimulated levels ofTGF-{3 are low in SLE patients, especially during active disease and the high IgG levels seen in those patients is attributed, in part, to low levels of TGF-{3 [30]. In animal models, TGF-{3 knockout mice were shown to develop SLE-like disease [31], whereas up-regulation of TGF-{3 production in the lupus prone *MRL/ipr/lpr* mice decreased autoantibody production [32]. Our previous observations also demonstrated that the beneficial effects in prevention and treatment of SLE in various animal models, following administration of the CDR-based peptides, were associated with up-regulation of TGF-{3 secretion [14].

It is not likely that the observed inhibition of the 16/6 Id-induced proliferative responses and IL-2 secretion by PBL of SLE patients is due merely to MHC blocking, because the effect of the peptides was specific (Figs 2 and 3) and the control peptides that were known to bind to MHC class II on APC of various SLE patients with a similar affinity (unpublished) to that of the CDR-based peptides [3] did not inhibit any of the above responses (Figs 2 and 3). Further, the CDR-based peptides stimulated specifically the secretion of the immunosuppressive cytokine TGF-{3 (Table 4, Fig. 3). These peptides might act as partial agonists stimulating a subset of PBL, such as CD4+CD25+ regulatory T cells that were shown to produce TGF-{3 [33] or by activating different signalling pathways aimed at the production of Immunosuppressive cytokines (e.g. TGF-{3) rather than the Th-1 type cytokines (e.g. IL-2). Indeed, in our previous studies the beneficial effects of the CDR-based peptides on two murine models of SLE were associated with a diminished secretion of the Th1-type cytokines and up-regulation of TGF-{3 production [13,14]. The fact that an elevated release of TGF-{3 could be triggered following incubation of PBL with the peptides themselves in the absence of the 16/6 Id-induced stimulation (Fig. 5) suggests that up-regulating the latter immunosupressive cytokine is a central step in the mechamism of action of the CDR-based peptides.

The peptides that are based on the CDR of the murine anti-DNA 16/6 Id autoantibody were shown previously by us to be capable of ameliorating experimental SLE in induced and spontaneous animal models [10,13-15]. Because of the observed high homology between various pathogenic anti-DNA antibodies [10] it is likely that our CDR-based peptides are capable of down-regulating other (non 16/6 Id) SLE-related aotoimmune responses. The amelioration of SLE-like disease in (NZB x NZW) F_1 mice by treatment with the CDR-based peptides [13] strengthens the latter assumption. A limited number of studies by other investigators also demonstraed beneficial effects on SLE manifestations mammal models by peptides based on variable regions of autoantibodies [34-37] or by synthetic peptides that can bind and block the reactivity of anti-DNA autoantibodies [38]. Recently, Hahn *et al.* reported the amelioration of SLE-like disease in (NZB x NZW) F_1 mice following treatment with a cosensus peptide based on amino acid sequences of murine anti-DNA MoAb probably via induction of tolerance in murine autoreactive T cells [39].

To conclude, we report here that, CDR1 to a lesser degree m.CDR3 and the newly synthesized peptides based on the an anti-DNA 16/6 Id autoantibody (hCDR1, hCDR3), mhlblted efficiently and specifically the *in vitro* 16/6 Id stimulation of PBL of SLE patients, apparently via a mechanism similar to that observed by us for the animal models. Thus, the above results suggest that these peptides might be novel potential candidates for specific treatment of SLE patients.

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REFERENCES

1 Winchester RJ. Systemic lupus erythematosus pathogenesis. In: Koopman, WJ, eds. Arthritis and allied conditions. Birmingham, Alabama: Williams and Wilkins, 1996:1361-91.

2 Isenberg DA, Shoenfeld Y, Madaw MP *et al.* Anti-DNA antibody IdIOtypes in systemic lupus erythematosus. Lancet 1984;2:417-22.

3 Dayan M, Segal R, Talsman A *et al.* Immune response of SLE patients to peptides based on the complementary determining regions (CDR) of a pathogenic anti-DNA monoclonal antibody. J Clin Immunol 2000; 20:187-94.

4 Mendlovic S, Shoenfeld Y, Bakimer R, Segal R, Dayan M, Mozes E. *In vitro* T-cell functions specific to an anti-DNA idiotype and serological markers in patients with systemic lupus erythematosus. J Clin Immunol 1988;8:178-87.

5 Mendlovic S, Segal R, Shoenfeld Y, Mozes E. Anti-DNA idiotype and anti-idiotype-specific T cell responses in patients with systemic lupus erythematosus and their first-degree relatives. Clin Exp Immunol 1990; 82:504-8.

6 Mendlovic S, Brocke S, Shoenfeld Y *et al.* Induction of a systemic lupus erythematosus-like disease in mice by a common human anti-DNA idiotype. Proc Natl Acad Sci USA 1988;85:2260--4.

7 Waisman A, Mendelovic S, Ruiz JP, Zinger H, Meshorer A, Mozes E. The role of the 16/6 idiotype network in the induction and manifestation of systemic lupus erythematosus. Int Immunol 1993; 5:1293-300.

8 Mendlovic S, Fricke H, Shoenfeld Y, Mozes E. The role of anti-idiotypic antibodies in the induction of experimental systemic lupus erythematosus in mice. Eur J Immunol 1989; 19:729-34.

9 Waisman A, Mozes E. Variable regions sequences of autoantibodies from mice with experimental systemic lupus erythematosus. Eur J Immunol 1993; 23:1566-73.

10 Waisman A, Ruiz PJ, Israeli E *et al.* Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of a pathogenic anti-DNA monoclonal antibody. Proc Natl Acad Sci USA 1997;94:4620-5.

11 Brosh N, Eilat E, Zinger H, Mozes E. Characterization and role in experimental systemic lupus erythematosus of T-cell lines specific to peptides based on complementarity-determining regions and complementarity-determining region-3 of a pathogenic anti-DNA monoclonal antibody. Immunology 2000;99:257-65.

12 Brosh N, Dayan M, Fridkin M, Mozes E. A peptide based on the CDR3 of an anti-DNA antibody of experimental SLE origin is also a dominant T-cell epitope in (NZB x NZW) F1 lupus-prone mice. Immunol Lett 2000; 72:61-8.

13 Eilat E, Zinger H, Nyska A, Mozes E. Prevention of systemic lupus erythematosus-like disease in (NZB x NZW) F1 mice by treating with CDR1- and CDR3-based peptides of a pathogenic autoantibody. J Clin Immunol 2000; 20:268-78.

14 Eilat E, Dayan M, Zinger H, Mozes E. The mechanism by which a peptide based on the complemetarity determining region-1 of a pathogenic anti-DNA autoantibody amehorates experimental SLE. Proc Natl Acad Sci USA 2001;98:1148-53.

15 Brosh N, Zinger H, Fridkin M, Mozes E. A peptide based on the sequence of the CDR3 of a murine anti-DNA MoAb is a better modulator of experimental SLE than its single amino acid-substituted analogs. Cell Immunol 2000;205:52-61.

16 Eilat E, Fridkin M, Mozes E. A peptide based on the CDRI of a pathogenic anti-DNA antibody is more efficient than its analogs in inhibiting autoreactive T cells. Immunobiology 2000; 202:383-93.

17 Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982; 25:1271-7.

18 Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum 1992; *35:630-40.*

19 Shoenfeld Y, Hsu-Lin SC, Gabriels JE *et al.* Production of autoantibodies by human human hybridomas. J Clin Invest 1982; 70:205-8.

20 Schnolzer M, Alewood PF, Kent SBH. *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int J Pept Protein Res 1992; 40:180-93.

21 Sthoeger ZM, Chiorazzi N, Lahita RG. Regulation of the immune response by sex hormones. I. *In vitro* effects of estradiol and testosterone on pokeweed mitogen-induced human B cell differentiation. J Immunol1988; 141:91-8.

22 Zisman E, Katz Levy Y, Dayan M *et al.* Peptide analogs to pathogenic epitopes of the human acetylcholine receptor alpha subunit as potential modulators of myasthenia gravis. Proc Natl Acad Sci USA 1996; 93:4492-7.

23 Williams WM, Staines NA, Muller S, Isenberg DA. Human T cell responses to autoantibody variable region peptides. Lupus 1995; 4:464-71.

24 Bermas BL, Petri M, Goldman D *et at.* T helper cell dysfunction in systemic lupus erythematosus (SLE): relation to disease activity. J Clin Immunol1994; 14:169-77.

25 Segal R, Bermas BL, Dayan M, Kalush F, Shearer GM, Mozes E. Kinetics of cytokine production in experimental systemic lupus erythematosus: involvement ofT helper cell Iff helper ce112-type cytokines in disease. J Immunol1997; 158:3009-16.

26 Funauchi M, Ikoma S, Enomoto H *et at.* Decreased Thl-like and increased Th2-like cells in systemic lupus erythematosus. Scand J Rheumatol1998; 27:219-24.

27 Vratsanos GS, Jung S, Park YM, Craft J. CD4+ T cells from lupus-prone mice are hyperresponsive to T cell receptor engagement with low and high affinity peptide antigens: a model to explain spontaneous T cell activation in lupus. J Exp Med 2001; 193:329-37.

28 Kitamura M, Suto T, Yokoo T, Shimizu F, Fmc LG. Transforming growth factor-beta 1 is the predominant paracrine inhibitor of macrophage cytokine synthesis produced by glomerular mesangial cells. J Immunol1996; 156:2964-71.

29 Horwitz DA, Gray JD, Ohtsuka K, Hirokawa M, Takahashi T. The immunoregulatory effects of NK cells: the role ofTGF-beta and implications for autoimmunity. Immunol Today 1997; 18:538-42.

30 Ohtsuka K, Gray JD, Stimmler MM, Toro B, Horwitz DA. Decreased production of TGF-beta by lymphocytes from patients with systemic lupus erythematosus. J Immunol1998; 160:2539-45.

31 Yaswen L, Kulkarni AB, Fredrickson T *et al.* Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. Blood 1996; 87:1439-45.

32 Raz E, Watanabe A, Baird SM *et at.* Systemic immunological effects of cytokine genes injected into skeletal muscle. Proc Natl Acad Sci USA 1993; 90:4523-7.

33 Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4 (+) CD25 (+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001; 194:629-44.

34 Singh RR, Ebling FM, Sercarz EE, Hahn BH. Immune tolerance to autoantibody-derived peptides delays development of autoimmunity in murine lupus. J Clin Invest 1995; %:2990--6.

35 Singh RR, Kumar V, Ebling FM *et al.* T cell determinants from autoantibodies to DNA can upregulate autoimmunity in murine systemic lupus erythematosus. J Exp Med 1995; 181:2017-27.

36 Kaliyaperumal A, Michaels MA, Datta SK. Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: tolerance spreading impairs pathogenic function of autoimmune T and B cells. J Immunol1999; 162:5775-83.

37 Jouanne C, Avrameas S, Payelle-Brogard B. A peptide derived from a polyreactive monoclonal anti-DNA natural antibody can modulate lupus development in (NZB x NZW) F1 mice. Immunology 1999; %:333-9.

38 Gaynor B, Putterman C, Valadon P, Spatz L, Scharff MD, Diamond B. Peptide inhibition of glomerular deposition of an anti-DNA antibody. Proc Natl Acad Sci USA 1997; 94:1955--60.

39 Hahn BH, Singh RR, Wong WK, Tsao BP, Bulpitt K, Ebling FM. Treatment with a consensus peptide based on amino acid sequences in autoantibodies prevents T cell activation by autoantigens and delays disease onset in murine lupus. Arthritis Rheum 2001; 44:432-41.

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Immunology

Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of a pathogenic anti-DNA monoclonal antibody

(autoantibodies/autoimmunity/immune tolerance/T-lymphocytes)

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ABSTRACT **Experimental systemic lupus erythematosus (SLE) can be induced in naive mice by immunization with a murine monoclonal anti-DNA antibody (mAb), 5G12, that bears a major idiotype designated 16/6 Id. Strain-dependent differences were observed in the proliferative responses of lymph node cells of mice immunized with two peptides based on the sequences of the complementarity determining region (CDR) 1 and 3 of mAb 5G12. The capacity of the peptides to bind to major histocompatibility complex class II molecules correlated with the proliferative responses. Immunization of high responder strains with the CDR-based peptides led to production of autoantibodies and clinical manifestations characteristic to experimental SLE. The CDR-based peptides could prevent autoantibody production in neonatal mice that were immunized later either with the peptide or with the pathogenic autoantibody. Furthermore, the peptides inhibited specific proliferation of lymph node cells of mice immunized with the same peptide, with mAb 5G12 or with the human mAb anti-DNA, 16/6 Id. Thus, the CDR-based peptides are potential candidates for therapy of SLE.**

Systemic lupus erythematosus (SLE) is a disease characterized by the production of autoantibodies to nuclear protein and nucleic acids, accompanied with clinical manifestations [e.g., leukopenia, thrombocytopenia, and kidney damage (1)]. We have previously demonstrated the induction of experimental SLE in naive mice of different strains following their inoculation with the human anti-DNA mAb carrying the 16/6 idiotype that was detected in sera of 54% SLE patients with active disease (2–4). The injected mice had high autoantibody levels that include anti-DNA and anti-nuclear protein antibodies, as well as 16/6 Id^+ and anti-16/6 Id antibodies, which indicate the activation of the 16/6 idiotypic network in those mice. We have further demonstrated that experimental SLE can be induced in mice of susceptible strains (BALB/c, SJL, and C3H.SW) following their immunization with either a murine anti-16/6 Id mAb (5) or a murine 16/6 Id^+ mAb, 5G12 (6). Experimental SLE, although induced in mice that normally do not develop any symptoms of SLE, was found to share features with (NZB × NZW)F_1 mice that develop the disease spontaneously. Thus, we have demonstrated high homology between the variable regions coding for the heavy and light chains of anti-DNA mAb isolated from mice afflicted with experimental SLE and the variable regions of anti-DNA mAb from (NZB × NZW)F_1 mice (7).

In the present article we report the synthesis and characterization of two peptides that were designed based on the sequences of the complementarity determining regions (CDRs) of a pathogenic murine monoclonal anti-DNA antibody (5G12) that bears the 16/6 Id. Those peptides were found to trigger T cell proliferation in various mouse strains, and induced, upon active immunization, the formation of autoantibodies and a mild experimental SLE in mice. Furthermore, when administrated in PBS into naive mice, the peptides were able to inhibit the production of autoantibodies and T cell activation in mice that were either immunized with these peptides or with the whole pathogenic antibody from a murine or human origin.

MATERIALS AND METHODS

Mice. The inbred mouse strains BALB/c and C57BL/6 were obtained from Olac (Oxon, U.K.). C3H.SW and SJL/J mice were obtained from The Jackson Laboratory. Female mice were used at the age of 8–10 weeks.

Synthetic Peptides. The CDR1-based peptide TGYYMQWVKQSPEKSLEWIG (pCDR1) and the CDR3-based peptide YYCARFLWEPYAMDYWGQGS (pCDR3) (the CDRs are underlined) were prepared with an automated synthesizer (Applied Biosystem model 430A) using the company's protocols for *t*-butyloxycarbonyl (BOC) strategy (8, 9). Peptide p307 of the human acetylcholine receptor α-subunit (10) and peptide p278 from the mouse heat shock protein 65 (11) were used as controls.

mAbs. The murine mAb 5G12 [IgG2a/κ; 16/6 Id^+ anti-DNA (6)] and 103 [IgG2a/κ; anti-(T,G)-A–L, (12)] were used. The human 16/6 anti-DNA mAb (IgG1/κ) was described (13, 14). Control human IgG were purchased from Sigma.

Immunization and Induction of Experimental SLE. Experimental SLE, was induced as described (3, 4). Briefly, mice were immunized with 1–2 μg of the human mAb 16/6, or with 10–20 μg of the murine mAb 5G12 or of the synthetic peptides emulsified in complete Freund's adjuvant (CFA). Three weeks later, the mice received a booster injection of the same dose of immunogen in aqueous solution.

Detection of SLE-Associated Clinical and Pathological Manifestations. Proteinuria was measured by a semiquantitative way, using Combistix kit (Bayer Diagnostics, Slough, U.K.). White blood cell were counted following a 10-fold dilution of the heparinized blood in distilled water containing 1% acetic acid (vol/vol). For immunohistology, frozen kidney sections (6 μm thick) were fixed and stained with fluorescein

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Abbreviations: APC, antigen-presenting cells; CDR, complementarity determining region; LNC, lymph node cells; MHC, major histocompatibility complex; SLE, systemic lupus erythematosus; CFA, complete Freund's adjuvant.

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isothiocyanate-conjugated goat antibodies to mouse immunoglobulin G (γ-chain specific; Sigma).

ELISA. ELISA was done as described (6). For coating of plates, the following antigen concentrations were used: 5 μg/ml of rabbit-anti-16/6-Id Ig, 10 μg/ml denatured calf thymus DNA (Sigma), 10 μg/ml HeLa nuclear extract (15), and 10 μg/ml of the human 16/6 Id mAb.

Proliferation Responses of Lymph Node Cells (LNC). LNC of immunized mice (0.5×10^6/well) were cultured in the presence of different antigens as described (6). Following 4 days of incubation, [^{3}H]thymidine (0.5 μCi of 5 Ci/mmol; 1 Ci = 37 GBq; Nuclear Research Center, Negev, Israel) was added. Sixteen hours later, cells were harvested and radioactivity was counted.

Direct Binding of Biotinylated Peptides to Antigen-Presenting Cells (APC). N-terminal biotinylation of the peptides was performed in 0.1 M sodium bicarbonate solution at room temperature, with excess of biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) (16). Binding of biotinylated peptides to spleen cells was done as described (17). Three antibodies were used for inhibition of binding: 34-5-3 (anti-I-A^b; PharMingen), MKD6 (anti-I-A^d; Becton Dickinson), and 10.3.6.2 [anti-I-A^s (18)].

Neonatal Tolerance. BALB/c female mice, 24 hr old, were injected i.p. with 50 μl of 2 mg/ml peptide in PBS. Forty-eight hours later, the mice received an additional i.p. injection of peptide. The latter were immunized at the age of 6–8 weeks with the peptide or mAb 5G12 in CFA, as described above.

Inhibition of LNC Proliferation. For inhibition of LNC proliferation, mice were injected i.p. with 200 μg of peptide in PBS. In most experiments injection of peptides as inhibitors was performed concomitant with the immunization. Ten days after the priming, LNC were collected and proliferation carried out as described above.

RESULTS

LNC Responses to the CDR-Based Peptides. To test whether peptides based on the sequences of the CDR of mAb 5G12 can trigger T cell reactivity, mice were immunized with pCDR1 and pCDR3, as well as with mAb 5G12. Ten days after immunization lymph nodes were removed and tested for proliferation. Table 1 shows the proliferative responses of four mouse strains. As can be seen in Table 1, LNC from SJL, BALB/c, and C3H.SW mice responded to mAb 5G12 by proliferation. LNC of BALB/c mice immunized with pCDR1 proliferated very strongly in the presence of pCDR1 (Table 1) but not in response to pCDR3. On the other hand, pCDR3 could induce a significant proliferative response in SJL and C3H.SW mice. LNC of C57BL/6 mice, the only mouse strain found to be completely resistant to SLE induction by both 16/6 Id and anti-16/6 Id mAb (4, 5), did not proliferate significantly to either mAb 5G12 or to the two CDR-based peptides. No significant stimulation above background (SI = 1–1.4) was observed when the LNC were incubated in the presence of a control peptide, namely p278. Further, LNC of mice injected with p278 did not proliferate to either mAb 5G12 or to pCDR1 and pCDR3.

Table 1. Proliferative responses of LNC taken from mice immunized with mAb 5G12 or with the CDR-based peptides

Mice immunized with	Mouse strains			
	SJL	BALB/c	C3H.SW	C57BL/6
mAb 5G12	21	8.2	11	2.2
pCDR1	1	69	16.5	1.3
pCDR3	6	1.1	4.7	1.4

Results are expressed as mean stimulation index (cpm following stimulation/background cpm) of triplicates, and represent one experiment out of four experiments performed.

Binding of the CDR-Based Peptides to Live APC. To test the ability of the CDR-based peptides to bind to major histocompatibility complex (MHC) class II molecules, the biotinylated peptides were employed in binding assays using live APC. As seen in Table 2, both peptides, pCDR1 and pCDR3, were capable of binding to live APC of C3H.SW, SJL or BALB/c mice. Although both CDR-based peptides were capable of binding to APC of C3H.SW mice (H-2^b), no significant binding was detected to APC of the H-2 matched strain C57BL/6 (Table 2). It is also shown in Table 2 that most of the binding of peptides pCDR1 and pCDR3 to live APC of the various mouse strains was inhibited by the relevant anti-I-A antibodies, but not by nonrelevant antibodies.

Induction of Lupus-Associated Autoantibodies and Clinical Manifestations by Immunization of Mice with the CDR-Based Peptides. It was of interest to find out whether SLE could be induced using the above peptides. To this end, naive BALB/c and SJL mice were immunized with pCDR1 and pCDR3. The mice produced high anti-peptide antibodies, specific to the injected peptide (data not shown). In agreement with the LNC proliferative responses (Table 1), BALB/c and SJL mice were high responders to the peptides pCDR1 and pCDR3, respectively, producing high levels of anti-single-stranded DNA antibodies (Fig. 1). The relevance of these peptides to the 16/6 Id is supported by the demonstration (Fig. 1*b*, BALB/c; and *d*, SJL) that mice immunized with the CDR-based peptides produced anti-16/6 Id antibodies, as compared with mice injected with a control peptide ($P < 0.008$ for BALB/c, and $P < 0.01$ for SJL), although this reaction is lower than that observed following injection with mAb 5G12 (Fig. 1). As previously reported for 16/6 Id and mAb 5G12 immunized mice (3, 6), neither binding to nonrelevant antigens (e.g., BSA) nor increase in total Ig levels could be measured in sera of the peptide immunized mice.

Mice immunized with the peptides were also tested for clinical symptoms of SLE. As seen in Table 3, BALB/c and SJL mice immunized with peptides pCDR1 and pCDR3, respectively, developed proteinuria and leukopenia. In addition, as shown in Fig. 2, these mice developed moderate levels of immune complex deposits in their kidneys in contrast to mice immunized with the control peptide p278.

Neonatal Tolerance Induction Using the CDR-Based Peptides. In an attempt to induce tolerance to the pCDR1 peptide, neonatal BALB/c mice were injected i.p. with 100 μg of the peptide in PBS 24 hr and 72 hr after birth. In parallel, BALB/c mice were injected with a nonrelevant peptide, p307 (10). The tolerized mice were immunized at the age of 6 weeks with either peptide pCDR1 or mAb 5G12.

As can be seen in Fig. 3, mice tolerized with pCDR1 and immunized with either peptide pCDR1 or mAb 5G12 showed

Table 2. Binding of the CDR-based peptides to MHC class II on live splenic APC

Mouse strain	% binding				% inhibition	
	pCDR1	pCDR3	H-2	mAb used	pCDR1	pCDR3
BALB/c	45	41	d	anti I-A^d (MKD6)	76.7	100
BALB/c			d	anti I-A^b (34-5-3)	0	0
SJL	42	43	s	anti I-A^s (10.3.6.2)	100	92.8
SJL			s	anti I-A^d (MKD6)	0	0
C3H.SW	42	53	b	anti I-A^b (34-5-3)	60	84.4
C3H.SW			b	anti I-A^d (MKD6)	0	25
C57BL/6	9	8.5	b			
C57BL/6			b			

Splenic adherent cells were incubated for 16 hr at 37°C with the biotinylated peptides in the presence or absence of anti-Ia mAb, stained, and analyzed thereafter as described.

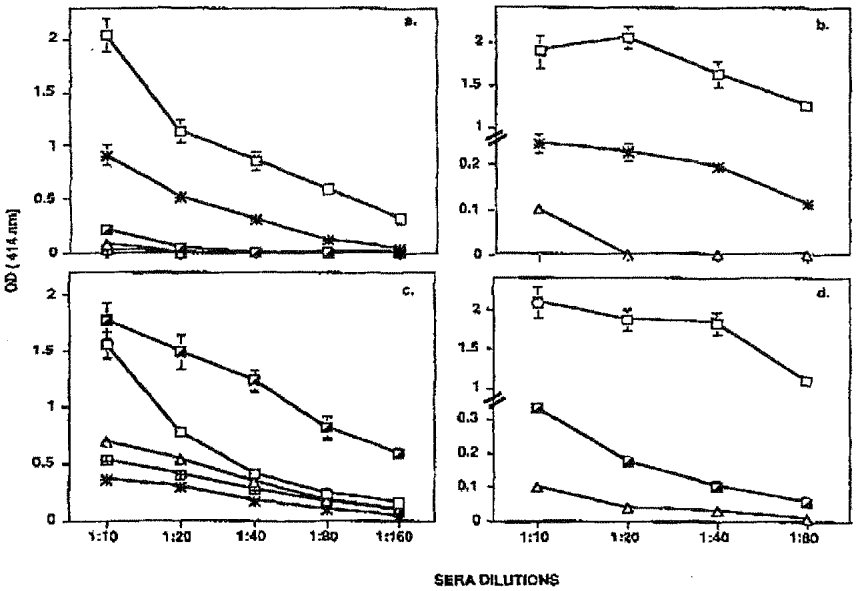

FIG. 1. Antibody levels in the sera of mice immunized with the CDR-based peptides. Sera of individual BALB/c (*a* and *b*) or SJL mice (*c* and *d*) immunized with either mAb 5G12 (□) or the peptides pCDR1 (*), pCDR3 (▨), or p278 (△) taken 3 months after the booster injection, and sera of age matched naive mice (⊞), were tested for anti-single-stranded DNA (*a* and *c*) and anti-16/6 Id (*b* and *d*) antibody titers. Results are expressed as mean OD ± SD of each mouse group. No significant background binding to uncoated plates (OD ≤ 0.05) could be detected with all sera tested.

reduced titers of antibodies to either DNA (Fig. 3*a*) or nuclear proteins (Fig. 3*b*), in comparison to the mouse groups that were tolerized with the control peptide, p307. In addition, neonatal tolerance with peptide pCDR1 greatly reduced the levels of 16/6 Id$^+$ antibodies in the sera of mice immunized with mAb 5G12, as compared with mice tolerized with the control peptide (Fig. 3*c*).

Inhibition of LNC Proliferation with the CDR-Based Peptides. Next, we attempted to inhibit proliferation of LNC of mature mice using the CDR-based peptides as inhibitors. As demonstrated in Fig. 4*a*, administration of 200 μg of peptide pCDR1 i.p. into BALB/c mice blocked 80–90% of the proliferative response to that peptide in mice that were immunized with the peptide in CFA. A similar inhibition was observed when the peptide was injected 3 days prior to the day of challenge, at the day of challenge or when the CDR-based peptide was given twice. Likewise, as seen in Fig. 4*b*, injection of SJL mice with pCDR3, i.p., in PBS, inhibited by 80–90% the capacity of LNC to proliferate to that peptide. The control peptide p307 did not affect the proliferative responses of LNC of these mice (Fig. 4).

To test the effect of the CDR-based peptides on the immune response to the whole murine anti-DNA 16/6 Id$^+$ mAb (5G12), BALB/c mice were injected with peptide pCDR1, in PBS, while SJL mice were injected with peptide pCDR3, concomitant with their immunization with mAb 5G12 in CFA. Fig. 5 shows that proliferative responses of LNC to the immunizing mAb were significantly reduced (60% inhibition) when pCDR1 was injected i.p. to BALB/c mice, or if peptide pCDR3 was injected i.p. in PBS to SJL mice (85% inhibition). LNC of mice immunized with mAb 5G12 proliferated also in response to the appropriate immunodominant peptide (pCDR1 for BALB/c and pCDR3 for SJL mice), a response that was completely reduced when the relevant peptide was injected concomitant with the immunization with the antibody. In contrast, coinjection of a nonrelevant peptide, p307, did not affect the proliferative response to mAb 5G12 in either mouse strain (Fig. 5).

We have previously demonstrated cross-reactivity on the level of T cell responses between murine and human mAbs bearing the 16/6 Id (6). Therefore, we tested the ability of the CDR-based peptides of the murine mAb 5G12 to modulate the T cell reactivity to the human mAb 16/6 Id. Mice were immunized with the human mAb 16/6 Id in CFA concomitant with an i.p. injection of either pCDR1, pCDR3, or p307. As depicted in Fig. 6*a*, injection of peptide pCDR1 to BALB/c

Table 3. Clinical manifestations of mice immunized with the CDR-based peptides

	BALB/c		SJL	
Immunization	WBC	Proteinuria	WBC	Proteinuria
mAb 5G12	3,800 ± 400	0.975 ± 0.08	ND	0.8 ± 0.07
pCDR1	3,375 ± 350	0.88 ± 0.076	ND	0.375 ± 0.04
pCDR3	3,325 ± 400	0.30 ± 0.01	3,300 ± 1,343	0.9 ± 0.075
p278	6,470 ± 920	0.33 ± 0.02	7,150 ± 320	0.2 ± 0.025
Nonimmunized	6,800 ± 1,200	0.1 ± 0	8,100 ± 475	0.05 ± 0

WBC (white blood cell) measured as counts per mm^3. Proteinuria was measured as g/liter. ND, not done.

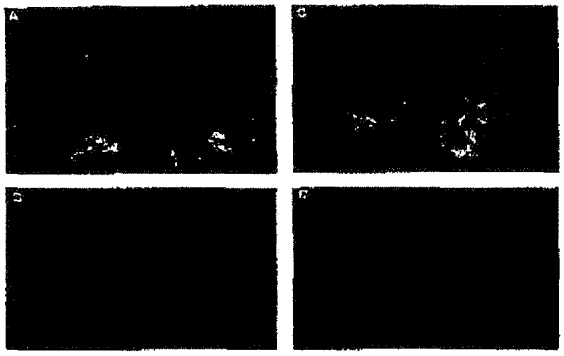

FIG. 2. Immunohistology of kidney sections from mice immunized with the CDR-based peptides. BALB/c (*A* and *B*) and SJL (*C* and *D*) mice were immunized with either pCDR1 (*A*), pCDR3 (*C*), or the control peptide p278 (*B* and *D*). Seven months later, mice were killed and their kidneys removed and analyzed for the presence of immune complex deposits as described. (×400.)

mice at the day of immunization with mAb 16/6, inhibited 90% of the proliferative response to the 16/6 Id. Similarly, pCDR3 inhibited the proliferative response of LNC of SJL origin to mAb 16/6 Id (Fig. 6*b*). Fig. 6 also demonstrates that LNC taken from BALB/c and SJL mice immunized with mAb 16/6 Id proliferated (SI of 2–4) in the presence of the immunodominant peptides of mAb 5G12, namely pCDR1 and pCDR3, respectively. The latter proliferative responses were also inhibited by the relevant CDR-based peptides.

DISCUSSION

In the present study, peptides based on the sequence of the CDRs of a pathogenic anti-DNA mAb (5G12) that bears the 16/6Id have been shown to be involved in both the induction of experimental SLE and the inhibition of the autoimmune responses.

Hahn and coworkers (19–21) found that a peptide of an anti-DNA mAb A6.1 isolated from NZB/W mice was involved in pathogenic and autoimmune processes in the mice. Thus, immunization of NZB/W mice with the latter CDR2 peptide, as well as with a cryptic peptide of the same antibody, increased plasma levels of anti-DNA IgG antibodies, accelerating immune complex deposition in the kidneys and nephritis (22, 23). The differences between the latter results and those of tolerance experiments reported here might be due to differences either in the peptides used or between the experimental model of induced SLE used by us and the spontaneous model of SLE studied by Hahn and coworkers (22, 23).

During the negative selection in the thymus, most of the T cells that recognize self-antigens are eliminated (24). Due to somatic mutations, peptides that represent the immunoglobulin CDR sequences are not always presented in the thymus, and as a consequence, T cells specific to those peptides may not be eliminated. A study utilizing a T cell line specific to a CDR2 peptide of a mAb showed that the line did not recognize the intact antibody (25). It was claimed that the cryptic CDR2 peptide is positively selected in the thymus (25). Indeed, the CDR2 in the latter antibody is encoded by a germ line gene sequence, and therefore exists in the thymus. In contrast, CDR1 and CDR3 of mAb 5G12 used in the present study are different from germ line gene sequences (7), and therefore it is highly probable that peptides pCDR1 and pCDR3 are not presented in the thymus.

Peptides pCDR1 and pCDR3 induced LNC proliferation in various mouse strains. Specifically, peptide pCDR1 was immunogenic in BALB/c mice, whereas peptide pCDR3 was immunogenic in SJL mice (Table 1). This pattern of proliferation may be explained by each of those peptides being the immunodominant peptides of mAb 5G12 in different mouse strains that are susceptible to the induction of experimental SLE, and may be the major contributors to the disease induction in that strain. The LNC taken from BALB/c mice that were immunized with mAb 5G12 were found also to proliferate significantly in the presence of peptides pCDR1 and pCDR3 (data not shown), suggesting that the mAb is processed to present at least those two peptides on the surface of APC. The latter is supported by our finding that MHC class II molecules on live APC isolated from C3H.SW, SJL, or BALB/c mouse strains were capable of binding both CDR-based peptides, pCDR1 and pCDR3 (Table 2).

LNC of C57BL/6 immunized mice did not proliferate to the peptides, and proliferated only weakly to mAb 5G12 (Table 1), while the H-2 matched mouse strain, C3H.SW, responded by proliferation to both CDR peptides and to mAb 5G12. In addition, in contrast to C3H.SW mice, APC isolated from C57BL/6 mice did not bind biotinylated pCDR1 and pCDR3. The latter may be due to a processing defect in mice of the

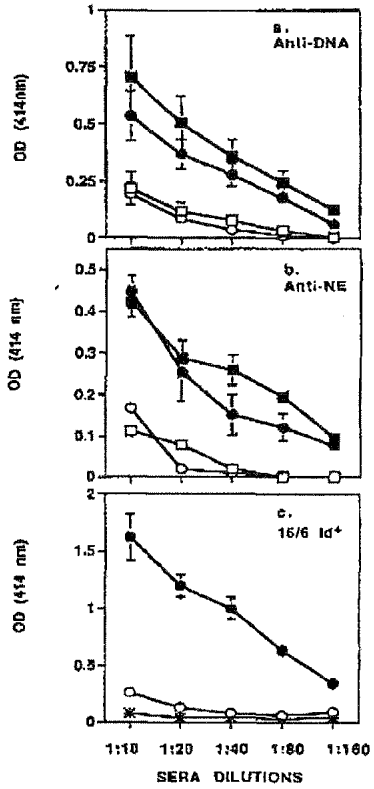

FIG. 3. Neonatal tolerization using the CDR-based peptides decrease autoantibody titers. Mice were tolerized and immunized as described. Two months following immunization, mice were bled and their sera subjected to ELISA experiments to test the levels of anti-single-stranded DNA (*a*), antinuclear antigens (*b*), and 16/6 Id$^+$ (*c*) specific antibodies. The groups include mice tolerized with peptide pCDR1 and immunized with either peptide pCDR1 (□) or with mAb 5G12 (○), mice tolerized with the nonrelevant peptide p307 and immunized with either peptide pCDR1 (■), or mAb 5G12 (●) and normal BALB/c mice (*). Results are expressed as mean OD ± SD.

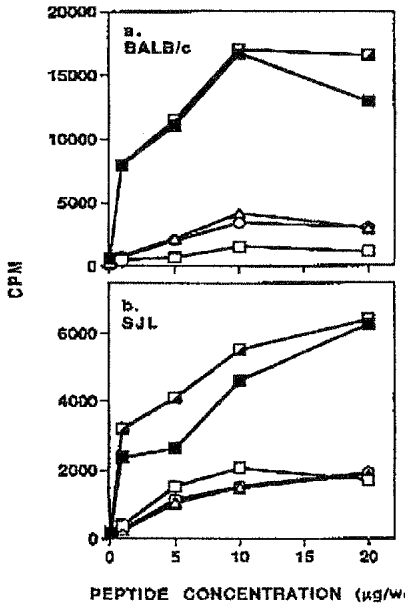

FIG. 4. *In vivo* inhibition of LNC proliferation. BALB/c (*a*) or SJL (*b*) mice were immunized i.d. in CFA with pCDR1 or pCDR3, respectively. The mice were also injected (i.p.) with 200 μg of the above immunizing peptides in PBS, either 3 days before immunization (□), at immunization day (○), or at both dates (△). Mice that were not treated (■), or treated with a control peptide, p307 (▨) were used as controls. LNC proliferation was then carried out as described. Results are expressed as mean cpm of triplicates. SD values did not exceed 10%.

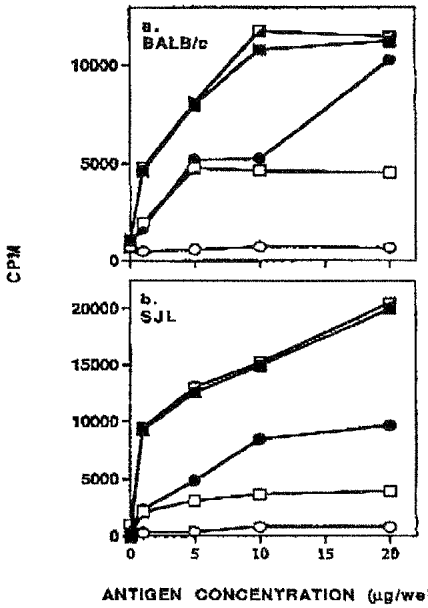

FIG. 5. LNC proliferative responses to mAb 5G12 in mice injected i.p. with the CDR-based peptides. LNC were taken from BALB/c (*a*) or SJL (*b*) mice treated with either pCDR1 (*a*) or pCDR3 (*b*). Proliferation is shown to mAb 5G12 of LNC taken from mice that were immunized and not treated (■), mice treated concomitant with immunization with peptide p307 (▨) or with the CDR-based peptides (□). Proliferation is also shown to the immunodominant CDR-based peptide of LNC taken from nontreated mice (●) or of mice treated with the peptide (○). LNC proliferation was then carried out as described. Results are expressed as mean cpm of triplicates. SD values did not exceed 10%.

C57BL/6 background, as was demonstrated recently for an hen egg lysozyme peptide (26). The inability of C57BL/6 to present the CDR-based peptides and to respond to the peptides by LNC proliferation may also explain the failure to induce experimental SLE in that mouse strain (4). It should be noted that as we have previously shown (17) APC of C57BL/6 mice are capable of binding efficiently a peptide that is a sequence of the human acetylcholine receptor α-subunit (p195–212). The resistance of the C57BL/6 mouse strain to the induction of experimental SLE was also observed following immunization with the human anti-Sm mAb 4B4, where anti-DNA antibodies were induced in BALB/c, but not in C57BL/6 mice (27). Thus, it appears that background genes of C57BL/6 can confer resistance to disease induction by autoantibodies.

It has been demonstrated that B cells are capable of presenting peptides from their own antibody to specific T cells on their MHC class II molecules (28, 29). LNC taken from SJL and C3H.SW mice immunized with pCDR1 were found to proliferate in the presence of peptide pCDR1 and also peptide pCDR3 (data not shown). We suggest, therefore, that following immunization with peptide pCDR1, B cells that produce an antibody that is similar or identical to the pathogenic mAb 5G12 may be activated. In turn, these B cells present a peptide similar or identical to pCDR3 and induce the formation of T cells specific to pCDR3. Therefore, in the induction of experimental SLE, the process of epitope spreading (30) may play an important role.

Peptides pCDR1 and pCDR3 induced autoantibody production as well as clinical manifestations including kidney damage. The latter corresponded to the ability of the peptides to trigger LNC to proliferate. These results may suggest that the initial trigger for induction of experimental SLE is a T cell response to the pathogenic moiety of the injected mAb.

In addition to the involvement of the CDR-based peptides in disease induction they were shown to be capable of inhibiting autoimmune responses. Thus, induction of tolerance to peptide pCDR1 in neonatal BALB/c mice inhibited autoantibody production following immunization with either peptide pCDR1 or mAb 5G12 (Fig. 3). Furthermore, administration of the CDR-based peptides in PBS could inhibit LNC proliferation to the peptides (Fig. 4), to the pathogenic parental murine mAb, 5G12 (Fig. 5), and to the original human anti-DNA 16/6 Id mAb (Fig. 6). The inhibition of the proliferation to the 16/6 Id may be due to the cross-reactivity on the T cell level between the murine 16/6 Id^+ mAb 5G12 and the human 16/6 mAb (6). The inhibition of serological manifestations as well as T cell proliferation by the CDR-based peptides suggest that they might be of potential for specific therapy of SLE.

Immunization by either pCDR1 or pCDR3 leads to the activation of peptide-specific T cells. Similarly, following injection of a pathogenic mAb, the latter is processed, and its CDR peptides are presented to specific T cells. This results in the formation of anti-peptide antibodies that in turn may cause the triggering of anti-self-antibodies and disease, or, the anti-peptide T cells induce directly the formation of anti-self-antibodies leading to disease.

Peptide pCDR1, when used to induce neonatal tolerance, could inhibit the formation of autoantibodies following immunization of BALB/c mice with either the peptide or mAb

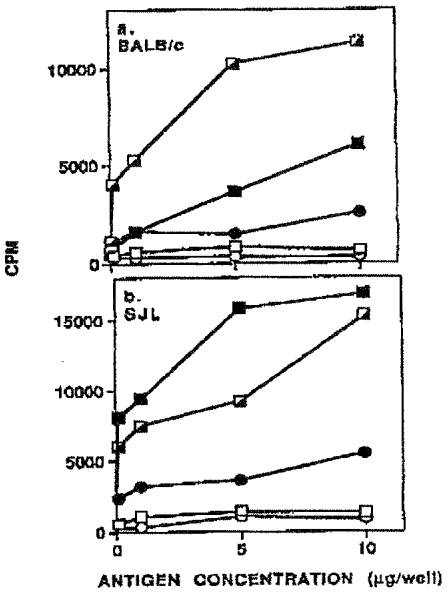

FIG. 6. LNC proliferative responses to the human mAb 16/6 Id in mice injected i.p. with the CDR-based peptides. Experiments were performed as described in the legend to Fig. 5.

5G12 (Fig. 3). The latter indicate that in BALB/c mice pCDR1 is a major T (Table 1) and possibly B cell epitope, and is a principal pathogenic moiety of the antibody. We also demonstrated that LNC proliferation to the dominant peptides or the whole antibody molecules could be abrogated by injection of the soluble peptides. The mechanism by which the soluble CDR-based peptides inhibited the LNC proliferation to the peptides or to the 16/6 Id^+ antibodies is not clear. We assume that the introduction of the immunodominant peptide in a soluble form inhibits the induction of newly activated T cells specific to it or to similar peptides, possibly by induction of anergy, blocking of epitope spreading, or shifting the cytokine profile of the activated T cells.

In conclusion, this study demonstrates that, without knowing the autoantigen for SLE, we have prepared peptides based on the CDR of a pathogenic autoantibody that can induce experimental SLE and also down regulate the autoimmune manifestations when used for tolerance in neonates or adult mice.

1. Shoenfeld, Y. & Mozes, E. (1990) *FASEB J.* 4, 2646–2651.
2. Isenberg, D. A., Shoenfeld, Y., Madaio, M. P., Rauch, J., Reichlin, M., Stollar, B. D. & Schwartz, R. S. (1984) *Lancet* ii, 417–422.
3. Mendlovic, S., Brocke, S., Shoenfeld, Y., Ben-Bassat, M., Meshorer, A., Bakimer, R. & Mozes, E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 2260–2264.
4. Mendlovic, S., Brocke, S., Fricke, H., Shoenfeld, Y. & Mozes, E. (1990) *Immunology* 69, 228–236.
5. Mendlovic, S., Fricke, H., Shoenfeld, Y. & Mozes, E. (1989) *Eur. J. Immunol.* 19, 729–734.
6. Waisman, A., Mendlovic, S., Ruiz, P. J., Zinger, H., Meshorer, A. & Mozes, E. (1993) *Int. Immunol.* 5, 1293–1300.
7. Waisman, A. & Mozes, E. (1993) *Eur. J. Immunol.* 23, 1566–1573.
8. Kent, S. B. H., Hood, L. E., Beilan, H., Meister, S. & Geiser, T. (1984) in *High Yield Chemical Synthesis of Biologically Active Peptides on an Automated Peptide Synthesizer of Novel Design*, ed. Ragnarsson, U. (Almqvist & Wiksell, Stockholm), pp. 185–188.
9. Schnolzer, M., Alewood, P. F. & Kent, S. B. H. (1992) *Int. J. Pept. Protein Res.* 40, 180–193.
10. Katz-Levy, Y., Kirshner, S. L., Sela, M. & Mozes, E. (1993) *Proc. Natl. Acad. Sci. USA* 90, 7000–7004.
11. Könen-Waisman, S., Fridkin, M. & I. R. Cohen (1995) *J. Immunol.* 154, 5977–5985.
12. Parhami-Seren, B., Eshhar, Z. & Mozes, E. (1983) *Immunology* 49, 9–19.
13. Shoenfeld, Y., Hsu-Lin, S. C., Gabriels, J. E., Silberstein, L. E., Furie, B. C., Furie, B., Stollar, B. D. & Schwartz, R. S. (1982) *J. Clin. Invest.* 70, 205–208.
14. Waisman, A., Shoenfeld, Y., Blank, M., Ruiz, P. J. & Mozes, E. (1995) *Int. Immunol.* 7, 689–696.
15. Waisman, A., Aloni, Y. & Laub, O. (1990) *Virology* 177, 737–744.
16. Zisman, E. & Mozes, E. (1994) *Int. Immunol.* 6, 683–691.
17. Mozes, E., Dayan, M., Zisman, E., Brocke, S., Licht, A. & Pecht, I. (1989) *EMBO J.* 8, 4049–4052.
18. Zamvil, S. S., Mitchell, D. J., Lee, N. E., Moore, A. C., Waldor, M. K., Sakai, K., Rothbard, J. B., McDevitt, H. O., Steinman, L. & Acha-Orbea, H. (1988) *J. Exp. Med.* 167, 1586–1596.
19. Tsao, B. P., Ebling, F. M., Roman, C., Panosian, S. N., Calame, K. & Hahn, B. H. (1990) *J. Clin. Invest.* 85, 530–540.
20. Tsao, B. P., Ohnishi, K., Cheroutre, H., Mitchell, B., Teitell, M., Mixter, P., Kronenberg, M. & Hahn, B. H. (1992) *J. Immunol.* 149, 350–358.
21. Tsao, B. P., Chow, A., Cheroutre, H., Song, Y. W., McGrath, M. E. & Kronenberg, M. (1993) *Eur. J. Immunol.* 23, 2332–2339.
22. Ebling, F. M., Tsao, B. P., Singh, R. R., Sercarz, E. E. & Hahn, B. H. (1993) *Arthritis Rheum.* 36, 355–364.
23. Singh, R. R., Kumar, V., Ebling, F. M., Southwood, S., Sette, A., Sercarz, E. E. & Hahn, B. H. (1995) *J. Exp. Med.* 181, 2017–2027.
24. Rudensky, A., Rath, S., Preston, H. P., Murphy, D. B. & Janeway, C. J. (1991) *Nature (London)* 353, 660–662.
25. Chen, J.-J., Kaveri, S.-V. & Kohler, H. (1992) *Eur. J. Immunol.* 22, 3077–3083.
26. Grewal, I. S., Moudgil, K. D. & Sercarz, E. E. (1995) *Proc. Natl. Acad. Sci. USA* 92, 1779–1783.
27. Dang, H., Ogawa, N., Takei, M., Lazaridis, K. & Talal, N. (1993) *J. Immunol.* 151, 7260–7267.
28. Bogen, B. & Weiss, S. (1989) *EMBO J.* 8, 1947–1952.
29. Weiss, S. & Bogen, B. (1991) *Cell* 64, 767–776.
30. Lehmann, P. V., Forsthuber, T., Miller, A. & Sercarz, E. E. (1992) *Nature (London)* 358, 155–157.

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Peptides based on the complementarity-determining regions of a pathogenic autoantibody mitigate lupus manifestations of (NZB × NZW)F_1 mice via active suppression

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Keywords: cytokine immunomodulation, epitope, experimental systemic lupus erythematosus, immunotherapy, *in vivo* animal model, peptide

Abstract

Two peptides based on the complementarity-determining regions (CDR) 1 and 3 (pCDR1 and pCDR3) of a murine monoclonal anti-DNA autoantibody that expresses the common idiotype 16/6Id were shown to down-regulate systemic lupus erythematosus (SLE)-associated T cell responses and to prevent the development of clinical symptoms in the SLE-prone mice, (NZB × NZW)F_1. In the present study the ability of the CDR-based peptides to treat an already established disease was tested. Mice were given 10 weekly injections of peptides either i.v. or s.c. The treatment led to a moderate reduction in the anti-DNA autoantibody titer, and a significant decrease in proteinuria and kidney pathology. The CDR-based peptides affected the pathogenic isotypes (IgG2a and IgG3) of the anti-DNA antibodies in the serum and in immune complexes in the kidneys. Both peptides mitigated disease manifestations and prolonged the survival of mice that were treated starting at the age of 7 months when full-blown disease was already developed. Furthermore, some beneficial effects of treatment with the CDR-based peptides could be adoptively transferred to diseased recipients. A reduction in the secretion of IL-2, IFN-γ, IL-4 and IL-10 was detected in supernatants of splenocytes of the treated mice. In contrast, treatment up-regulated the immunosuppresive cytokine-transforming growth factor-β. Thus the ameliorating effect of the CDR-based peptides on SLE manifestations is at least partially via the immunomodulation of the cytokine profile.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the increased production of autoantibodies and defective T cell-mediated responses. The latter are associated with various clinical manifestations including immune complex depositions in the kidneys and other organs (1). Female (NZB × NZW)F_1 mice develop spontaneously an SLE-like syndrome. Beginning at the age of ~3–4 months, autoantibodies, including anti-DNA antibodies, are developed in these mice and, by 7–8 months, immune complex deposits form in the kidneys. Mice die of the disease within 1 year (2).

Our previous studies have demonstrated that experimental SLE can be induced in mice of susceptible strains (e.g. BALB/c, SJL, C3H.SW) following their immunization with anti-DNA monoclonal autoantibodies that express a major idiotype designated 16/6Id of either human or mouse origin (3,4). Experimental SLE, although induced in mice that normally do not develop any symptoms of SLE, was found to share features with the disease of (NZB × NZW)F_1 mice that develops spontaneously. Thus, high homology was demonstrated between the variable regions coding for the heavy and light chains of anti-DNA mAb isolated from mice afflicted with experimental SLE and the variable regions of anti-DNA mAb from (NZB × NZW)F_1 mice (5–7).

We have previously shown that two peptides based on the sequences of the complementarity-determining regions (CDR) 1 and 3 of a murine anti-DNA 16/6Id$^+$ mAb designated mAb

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5G12 (8) were capable of inhibiting specific proliferation of lymph node cells of mice immunized with the same peptide, with the murine mAb 5G12 or with the human mAb anti-DNA, 16/6Id. Furthermore, the CDR-based peptides could prevent autoantibody production in neonatal mice that were immunized later either with the peptide or with the pathogenic autoantibody. We have recently shown that pCDR1 administered in PBS could either prevent or down-regulate experimental SLE induced in naive mice by affecting the production of autoantibodies and the severity of the clinical manifestations, including kidney damage (9). Moreover, in a previous work we have reported that the CDR-based peptides could prevent the spontaneous development of SLE in (NZB × NZW)F_1 mice. Both peptides, pCDR1 and pCDR3, given in PBS to 2-month-old (NZB × NZW)F_1 mice reduced the levels of anti-DNA autoantibodies and the clinical manifestations, including kidney damage, in the treated mice (10).

The above line of evidence prompted us to investigate whether the CDR-based peptides might be of therapeutic benefit in the treatment of an already established clinical SLE-like disease in (NZB × NZW)F_1 mice. We report here that both peptides, pCDR1 and pCDR3, given in PBS to (NZB × NZW)F_1 mice with lupus-like manifestations down-regulated autoantibody production as well as clinical symptoms. The beneficial effects of treatment were associated with a diminished production of the T_h1-type cytokines (IL-2 and IFN-γ) as well as T_h2-type cytokines (IL-4 and IL-10), whereas secretion of the immunosuppressive cytokine transforming growth factor (TGF)-β was elevated. Further, the beneficial effects of treatment could be transferred to diseased recipients by splenocytes of young (2-month-old) mice that were treated with either pCDR1 or pCDR3. Up-regulated levels of TGF-β could be detected in supernatants of splenocytes of peptide-treated mice.

Methods

Mice

Female (NZB × NZW)F_1 mice (6–8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under standard conditions in the animal facility of The Weizmann Institute of Science.

Synthetic peptides

The peptides based on the CDR of the murine anti-DNA, 16/6Id mAb (5G12) were used in the study. The CDR1-based peptide TGYYMQWVKQSPEKSLEWIG (pCDR1) and the CDR3-based peptide YYCARFLWEPYAMDYWGQGS (pCDR3) (the CDR are underlined) were synthesized with an automated synthesizer (model 430A; Applied Biosystems, Weiterstadt, Germany) using the company's protocols for the t-BOC strategy (11). A peptide synthesized in the reversed order of pCDR1 (GIWELSKEPSQKVWQMYYGT) was used for control.

Treatment of mice with the CDR-based peptide

Groups of (NZB × NZW)F_1 mice at the age of 5–7 months were treated with either pCDR1 or pCDR3. Control groups were treated with either the vehicle, PBS, only or with the control peptide, reversed pCDR1. Various concentrations (100, 250 or 500 μg/mouse) of the peptides were administered into the mice in PBS either i.v. or s.c., once a week for 10 weeks.

Transfer of spleen cells from animals treated with the CDR-based peptides

(NZB × NZW)F_1 female mice (8–10 weeks old) received three s.c. injections (every other day) of 300 μg of pCDR1, pCDR3 or the control reversed pCDR1. Thereafter, spleen cells were harvested and injected i.p. (20×10^6/mouse) into 8-month-old (NZB × NZW)F_1 recipients. The mice were followed for the presence of dsDNA-specific antibodies, proteinuria and immune complex deposits.

ELISA

For measuring anti-dsDNA antibodies, 96-well Maxisorb microtiter plates (Nunc, Roskilde, Denmark) were coated with poly-L-lysine (Sigma, St Louis, MO). The plates were then washed and coated with λ phage dsDNA (Worthington Biochemical, Lakewood, NJ). After incubation with different dilutions of sera, goat anti-mouse IgG (γ chain specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) was added to the plates. For the determination of anti-DNA antibodies of various Ig isotypes, horseradish peroxidase conjugated to goat anti-mouse γ1, γ2a, γ2b or γ3 chain-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) was used. Plates were then incubated with the substrate, ABTS [2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); Sigma] and read at 405 nm using an ELISA reader.

Detection of proteinuria

Proteinuria was measured by a standard semi-quantitative test, using an Albustix kit (Bayer Diagnostic, Newbury, UK). Results were graded according to the manufacturer as: negative, + = 0.3g/l, ++ = 1g/l, +++ = 3g/l or ++++ = ⩾20g/l.

Immunohistology

Mice were sacrificed at the age of 8–9 months, and kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections (5 μm) were air-dried and fixed in acetone. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (Jackson ImmunoResearch). For the determination of the various Ig isotypes FITC-conjugated goat anti-mouse IgG2a (γ2a chain specific) or IgG3 (γ3 chain specific) antibodies (Southern Biotechnology Associates) were used. Staining was visualized using a fluorescence microscope.

Histopathology

Mice were sacrificed and their kidneys were preserved frozen at −70°C, and then trimmed and routinely processed for light microscopy. Paraffin-embedded, 5-μm thick sections were stained with hematoxylin & eosin. Lesions were scored using semiquantitative grading as follows: 0 = no lesions, 1 = minimal lesions, 2 = mild lesions, 3 = moderate lesions and 4 = severe lesions. Histopathology was evaluated with the pathologist blinded to whether mice belonged to treated or untreated groups.

Table 1. Comparison of the effects of the i.v. and s.c. treatment protocols[a]

Treatment	Anti-dsDNA (OD ± SEM)[b]	Proteinuria (g/l ± SEM)[c]	Average intensity of immune complex deposits (± SEM)[d]
Untreated	0.89 ± 0.1	9.74 ± 1.14	2 ± 0.18 (10)[m]
pCDR1 100 µg i.v.	0.58 ± 0.1	0.45 ± 0.4[e]	1.3 ± 0.8[g] (10)
pCDR1 250 µg i.v.	0.87 ± 0.1	1.36 ± 1.26[f]	0.3 ± 0.5[h] (9)
PBS s.c.	1.10 ± 0.2	11 ± 3.01	2.4 ± 0.3 (10)
pCDR1 100 µg s.c.	0.88 ± 0.1	0.84 ± 0.42[i]	0.88 ± 0.35[k] (9)
pCDR1 250 µg s.c.	0.74 ± 0.1	2.69 ± 1.96[j]	1.05 ± 0.33[l] (10)

[a]Representative of two experiments.
[b]Dilution of sera 1:250. Results are of sera from mice that were bled after the end of treatment.
[c]Proteinuria was always measured at about the same time of day and all mice in an experimental cohort were tested together. Results are of mice at the age of 7 months.
[d]Immune complex deposits were assessed at sacrifice when mice reached the age of 8 months.
[e–h]$P < 0.01$ as compared to the untreated group.
[i,j]$P < 0.01$, [k,l]$P < 0.04$ as compared with the PBS treated group.
[m]Numbers in parentheses are of animals/group and apply for all evaluated manifestations.

Cytokine production by splenocytes

Spleen cells (5×10^6/ml) of the tested mice were incubated with either enriched medium only or concanavalin A (Con A; 2.5 µg/ml).Supernatants were removed after 24 h and analyzed for cytokine content. IL-2, IFN-γ, IL-4 and IL-10 were determined by ELISA using OptEIA sets (PharMingen, San Diego, CA) and according to the manufacturer's instructions. For detection of TGF-β, plates were coated with a recombinant human TGF-β sRII/Fc chimera. Supernatants were added after activation of latent TGF-β1 to immunoreactive TGF-β1 according to the manufacturer (R & D Systems) A standard recombinant TGF-β1 was used as well. Thereafter, a biotinylated anti-human TGF-β1 antibody was added and the assay was developed according to the manufacturer's instructions (R & D Systems).

Detection of intracellular cytokines

Single-cell suspensions of spleens were exposed to a Cytoperm kit (Serotec, Oxford, UK) according to the company's protocol. Thereafter, cells were incubated with the appropriate anti-cytokine–FITC conjugated antibody. Cells were assessed by a FACScan cytometer and the data was analyzed using Lysys software.

Statistical analysis

To evaluate the significance of the difference between untreated and treated groups, the Student's *t*-test and the non-parametric Mann–Whitney test were used. Values of $P \leq 0.05$ were considered significant.

Results

Effect of treatment with pCDR1 using different routes

We have previously shown that treatment of (NZB × NZW)F_1 mice by administration of either pCDR1 or pCDR3 at the age of 2 months either prevented the development of the SLE-like disease or resulted in a milder disease in the treated mice (10). It was of interest to find out whether treatment of (NZB × NZW)F_1 mice at the age of 5 months, when SLE clinical symptoms are already detected, will affect the disease course. To this end, (NZB × NZW)F_1 mice at the age of 5 months were injected with pCDR1 (100 or 250 µg/mouse) either i.v. or s.c., once a week for 10 weeks. A group of mice was treated similarly with the vehicle only. Animals were monitored for the presence of anti-dsDNA autoantibodies, levels of protein in the urine (proteinuria) and immune complex deposits in their kidneys. Table 1 demonstrates representative results of such an experiment. As shown in Table 1, i.v. treatment with 100 µg/mouse of pCDR1 resulted in a moderate reduction in anti-dsDNA antibody titers. Subcutaneous treatment either with 100 or 250 µg/mouse led to a similar reduction in the anti-DNA autoantibody titers (Table 1). Both administration routes led to a significant reduction in proteinuria and in the immune complex deposits in the kidneys of the pCDR1-treated mice (Table 1). Thus, analysis indicated that 50–55% of the kidneys in the groups of pCDR1-treated mice were completely free of immune complex deposits. Kidneys of the additional mice in the treated groups had immune complexes of lower intensity than in the control groups. Thus, both routes of administration of the pCDR1 are similarly effective in down-regulating the clinical symptoms of the SLE-like disease in the (NZB × NZW)F_1 mice. Therefore, in further experiments the s.c. route was used.

Treatment of mice by s.c. administration of pCDR3 at a dose of either 100 or 250 µg/mouse ameliorated the clinical manifestations similarly to pCDR1. Thus, levels of proteinuria were lower by 75–90% of those in the PBS-treated groups. Furthermore, in comparison to average intensity of immune complex deposits of 2 ± 0.37 in the group of PBS-treated mice, values of 1 ± 0.27 ($P = 0.02$) and 1 ± 0.42 ($P = 0.04$) were determined for kidneys in the groups of mice treated with 100 and 250 µg/mouse respectively.

Peptides pCDR1 and pCDR3 ameliorate specifically the SLE-like disease in (NZB × NZW)F_1 mice

The specificity of the beneficial effects of pCDR1 and pCDR3 was also tested. Thus, groups (10 mice per group) of 5-month-old (NZB × NZW)F_1 mice were treated s.c. with 250 µg/mouse of the CDR-based peptides or with a control peptide (reversed pCDR1) weekly for 10 weeks. As can be seen in Table 2, a decrease in anti-dsDNA antibody titer was determined in the

Table 2. Treatment with the CDR-based peptides down-regulates specifically the clinical manifestations in (NZB × NZW)F_1 mice[a]

Treatment	Anti-dsDNA (OD ± SEM)[b]	Proteinuria (g/l ± SEM)[c]	Average intensity of immune complex deposits (± SEM)[d]
Untreated	1.12 ± 0.2	10.32 ± 5.59	2.40 ± 0.4
Reversed CDR1 250 μg	1.17± 0.1	10.32 ± 3.66	2.75 ± 0.25
pCDR1 250 μg	0.59 ± 0.1[e]	0.94 ± 0.53[f]	1.60 ± 0.4[h]
pCDR3 250 μg	0.85 ± 0.2	1.04 ± 0.45[g]	1.33 ± 0.49[i]

[a]Representative of two experiments.
[b]Dilution of sera 1:250 of mice that were bled 1 month after the end of treatment.
[c]Proteinuria was measured at the age of 7 months.
[d]Mice were sacrificed at the age of 8 months and their kidneys were analyzed.
[e–i]$P < 0.05$ as compared with the control groups.

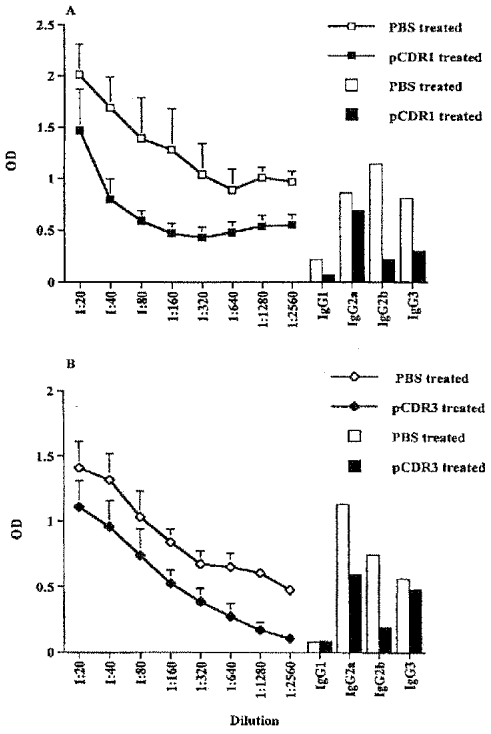

Fig. 1. Reduction of anti-DNA autoantibody titers. (NZB × NZW)F_1 mice were treated with either PBS or the CDR-based peptides (A) pCDR1 or (B) pCDR3 starting at the age of 5 months (s.c., 250 μg/mouse). Mice were bled monthly and sera of individual mice were tested for anti-DNA antibody titer [total IgG and the various isotypes (sera dilution 1:40) as described in Methods]. Results are of sera of mice that were bled shortly after treatment and are representatives of three individual experiments.

sera of the treated mice as compared to either untreated mice or to mice that were treated with the reversed pCDR1. Table 2 also shows that the treatment with the CDR-based peptides significantly decreased the levels of proteinuria and severity of immune complex deposits in the kidneys ($P < 0.05$ for both pCDR1 and pCDR3 treatment protocols as compared to the untreated and reversed pCDR1-treated groups).

Treatment of (NZB × NZW)F_1 mice with pCDR1 and pCDR3 down-regulates the pathogenic isotypes of the anti-DNA autoantibodies

It was of interest to find out whether the reduction in anti-DNA autoantibody titers could be attributed to one or more of the IgG isotypes. Therefore, in addition to the total anti-DNA antibody detected in sera of individual mice, the sera were tested for the presence of anti-DNA antibodies of the various isotypes. Representative results are shown in Fig. 1. It can be seen in Fig. 1(A) that treatment with pCDR1 resulted in a slight decrease in anti-DNA antibodies of the IgG2a isotype, whereas the decrease in antibody titers of the IgG2b and IgG3 isotypes was much more pronounced. Figure 1(B) shows that treatment with pCDR3 led to a decrease mainly in the IgG2a and IgG2b antibody isotypes, and a very mild decrease in the IgG3 isotype. Similar results were obtained in three independent experiments.

Immunohistology examination of the immune complex deposits in kidneys of mice that were treated with the CDR-based peptide showed that the reduction in total IgG immune complex deposits could be also observed in the immune complexes of IgG2a and IgG3. Thus, it can be seen in Fig. 2 that treatment with pCDR1 (Fig. 2B) and pCDR3 (Fig. 2C) reduced the intensity of immune complex deposits, as compared to treatment with PBS (Fig. 2A), when the latter were assessed with antibodies to total IgG as well as with antibodies to IgG2a and IgG3.

Histopathological evaluation of kidneys of untreated (NZB × NZW)F_1 mice and of mice treated with the CDR-based peptides

The histopathological findings are presented in Table 3 and Fig. 3. Three components of renal pathological changes were evaluated: glomerulosclerosis, nephropathy and interstitial lymphocytic infiltration. Glomerulosclerosis was diagnosed when the glomerular basement membrane appeared thickened. The process involved hyaline obliteration of the glomeruli, transforming them into acellular ecsinophilic masses (crescentic formation). Nephropathy diagnosis was determined when the renal tubules appeared atrophic or

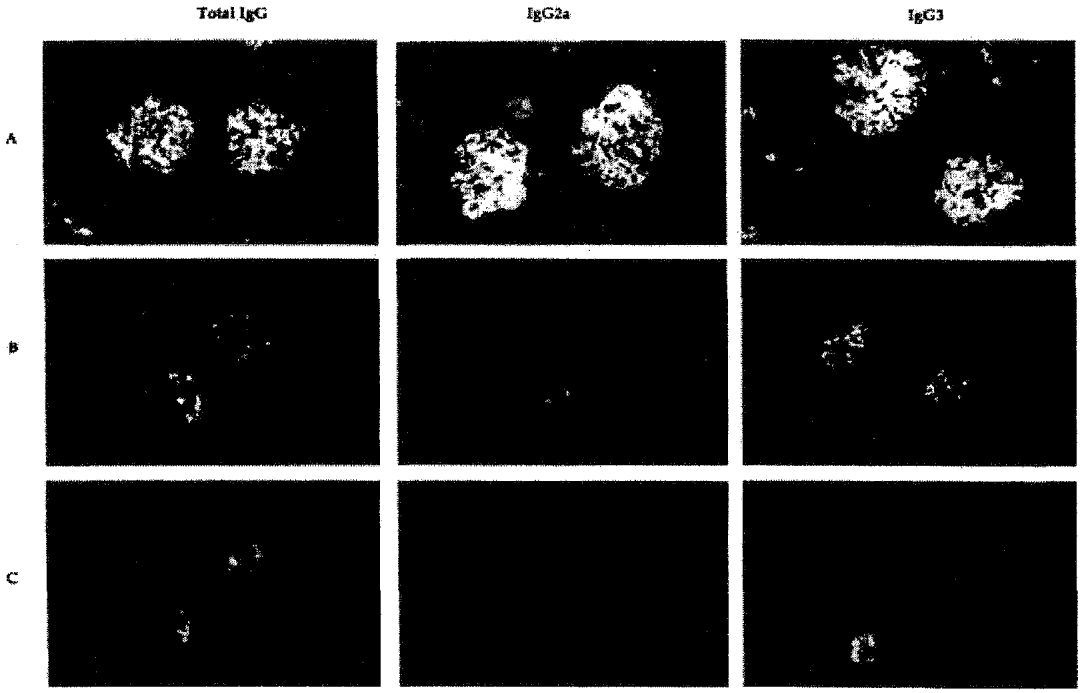

Fig. 2. Immunohistology examination of immune complex deposits of total and IgG isotypes in kidneys of (NZB × NZW)F_1 mice that were treated with the CDR-based peptides. Frozen cryostat sections (5 μm) of 8-month-old (NZB × NZW)F_1 mice were air dried, fixed in acetone and stained with FITC-conjugated to goat anti-mouse IgG (γ chain specific), goat anti-mouse IgG2a (γ2a chain specific) or goat anti-mouse IgG3 (γ3 chain specific). Representative kidney sections of (A) PBS-treated mice, (B) pCDR1-treated mice and (C) pCDR3-treated mice (×400).

Table 3. Histopathological findings in kidneys of (NZB × NZW)F_1 mice

Treatment	Glomerulosclerosis	Nephropathy	Interstitial lymphocytic infiltration
PBS	2.15 ± 0.38	2.54 ± 0.43	1.38 ± 0.33
pCDR1 250 μg	1.00 ± 0.35[a]	1.22 ± 0.35[c]	0.30 ± 0.21[e]
pCDR3 250 μg	0.88 ± 0.30[b]	1.22 ± 0.36[d]	0.33 ± 0.23[f]

Histopathological findings: paraffin-embedded, 5-μm thick sections were stained with hematoxylin & eosin. Histopathological findings were evaluated in three components of renal pathological changes: glomerulosclerosis, nephropathy and interstitial lymphocytic infiltration.

Microscopic evaluation: histopathological changes were graded on a scale of 0–4 and in ascending order, according to the degree of severity as shown: 0 = unremarkable, 1 = minimal, 2 = mild, 3 = moderate and 4 = marked changes. For final evaluation, the mean value for severity of each observed lesion was calculated.

[a,b]$P < 0.02$; [c,d]$P < 0.04$; [e,f]$P < 0.03$ as compared with the PBS-treated group.

regenerative, basophilic, having dilated lumen with presence of proteinaceous casts. The term interstitial lymphocytic infiltration was applied to define the presence of scattered and discrete interstitial lymphocytic and other mononuclear cell aggregations, present at the medullary regions. Increased severity of glomerulosclerosis and nephropathy was accompanied by dissemination of lymphocytic infiltration within cortical and medullary regions. The mean severities of the histopathological findings are presented in Table 3. The incidence and, in particular, the severity of these parameters were significantly reduced in the groups treated with the peptides, as compared to the group treated only with PBS. In particular, the severities of glomerulosclerosis and nephropathy, which are characteristic components of the spontan-

eous glomerulonephritis in (NZB × NZW)F_1 mice, were prominently reduced in the pCDR1- and pCDR3-treated mice (Table 3). Figure 3 demonstrates kidney sections of mice that were given the vehicle, PBS, only (Fig. 3A and B) and of mice treated s.c. with 10 weekly injections of either pCDR1 (Fig. 3C) or pCDR3 (Fig. 3D). Figure 3(B) represents a

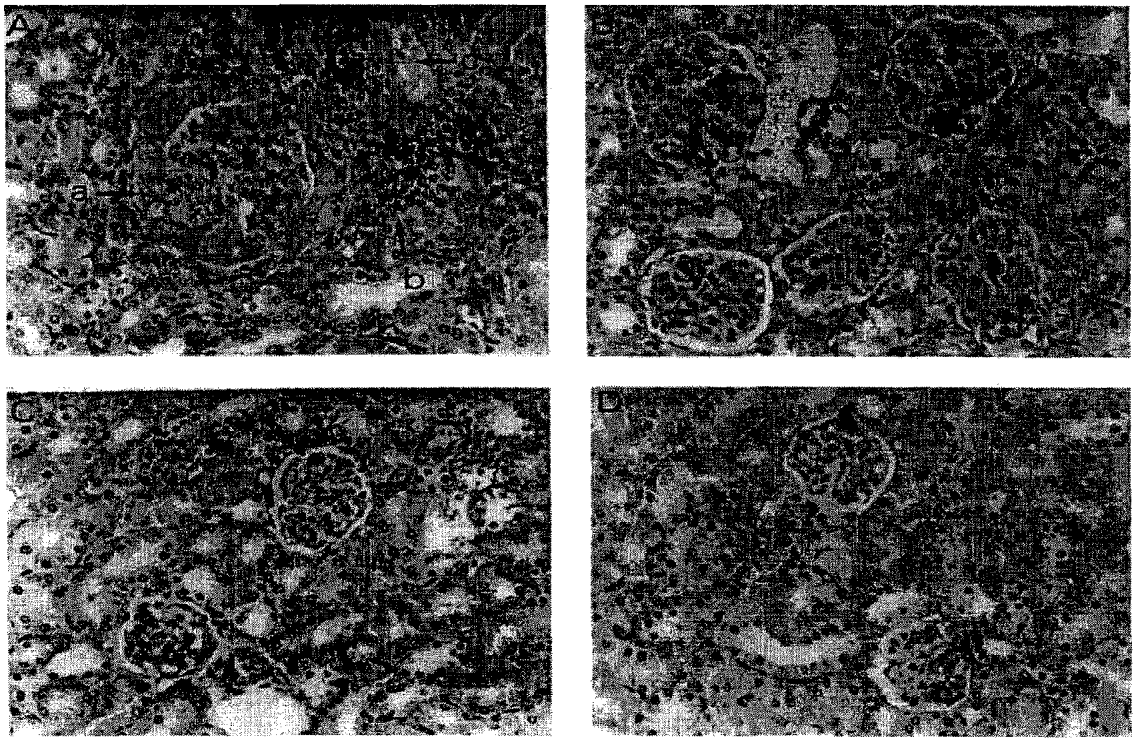

Fig. 3. Histopathology of kidney sections of (NZB × NZW)F_1 mice untreated or treated with the CDR-based peptides. Groups (10 mice per group) of 5-month-old (NZB × NZW)F_1 mice were treated s.c. with PBS only (controls) or with 250 μg/mouse of either pCDR1 or pCDR3 in PBS. The mice were treated once a week for 10 weeks. Paraffin-embedded, 5-μm thick sections of kidneys of mice that were sacrificed at the age of 8 months (~2 weeks after the end of treatment) were stained with hematoxylin & eosin. (A) A kidney section of a mouse given the vehicle, PBS, only. Immune glomerulonephritis. (a) Cresentic formation: thickening of Bauman's capsule and glomerulosclerosis. (b) Interstitial nephritis (lymphocytes and other mononuclear cells). (c) Protein casts in the lumen of the tubuli. Late stage. (B) A kidney section of a mouse given the vehicle, PBS, only. Mesangial cell proliferation (mesangial proliferative glomerulonephritis). Early stage. (C) A kidney section of a mouse that was treated with the CDR1-based peptide. Normal pattern of kidney tissue. (D) A kidney section of a mouse that was treated with the CDR3-based peptide. Normal pattern of kidney tissue. Hematoxylin & eosin (×350).

Table 4. Treatment of 7-month-old (NZB × NZW)F_1 mice with the CDR-based peptides reduces the clinical manifestations of their SLE-like disease

Treatment	Anti- dsDNA (OD ± SEM)[a]	Proteinuria (g/l ± SEM)[b]	Intensity of immune complex deposits (± SEM)[c]	Survival [*n* (%)]
PBS treated	1.49 ± 0.3	12.53 ± 3.06	2.25 ± 0.25	4/11 (36%)
pCDR1 250 μg	1.07 ± 0.1	1.80 ± 0.41[d]	1.00 ± 0.37[g]	8/10 (80%)[j]
pCDR1 500 μg	1.01 ± 0.2	4.41 ± 2.27	1.40 ± 0.6	5/10 (50%)
pCDR3 250 μg	1.02 ± 0.1	1.17 ± 0.45[e]	0.77 ± 0.32[h]	9/11 (82%)[k]
pCDR3 500 μg	0.91 ± 0.1	0.65 ± 0.35[f]	0.75 ± 0.31[i]	8/10 (80%)[l]

[a]Results are of sera from mice that were bled 1 month after the end of treatment. Dilution of sera 1:250.
[b]Proteinuria was always measured at about the same time of day and all mice in an experimental cohort were tested together. Results are of mice at the age of 8 months.
[c]Immune complex deposits were assessed at sacrifice when mice reached the age of 9.5 months.
[d-l]$P < 0.02$; [e-l]$P < 0.04$; [f-l]$P < 0.05$ as compared with the PBS-treated group.

kidney of a mouse with early stages of glomerulonephritis characterized by mesangial cell proliferation, whereas Fig. 3(A) depicts a kidney section of a mouse with a severe kidney damage: (a) glomerulosclerosis, (b) interstitial nephritis and (c) cast formation. The histological analysis of kidneys of mice treated with the CDR-based peptides (Fig. 3C and D) shows a normal pattern with no morphological changes.

Treatment of 7-month-old (NZB × NZW)F_1 mice with the CDR-based peptides ameliorated SLE manifestations at the advanced stage

We have further tested the ability of both CDR-based peptides to mitigate the SLE-like manifestation in aged (NZB × NZW)F_1 mice that suffer already from severe clinical manifestations. To this end 7-month-old (NZB × NZW)F_1 mice in which high levels of dsDNA autoantibodies and high proteinuria have been detected were treated with the peptides once a week for 10 weeks by the administration of either 250 or 500 μg/mouse s.c. in PBS. Control mice were treated with PBS alone. Table 4 shows that, in addition to the observed reduction in dsDNA-specific antibody levels in all treated groups, lower proteinuria levels were measured as well. Beneficial effects were also observed when immune complex deposits were determined as demonstrated in Table 4. It is also shown in Table 4 that the survival rate was significantly higher in the groups treated with 250 μg of pCDR1 and with 250 and 500 μg of pCDR3 when compared to PBS-treated animals. Thus, >60% of the PBS-treated mice died before the last injection, whereas most of the treated mice survived and either were healthy or had mild disease manifestations. It is noteworthy that in the present experiment mice were treated with the 250 μg dose that was used in most experiments and with a higher dose of 500 μg/mouse. Nevertheless, the results indicate that the 250 μg dose is efficient enough in ameliorating disease manifestations.

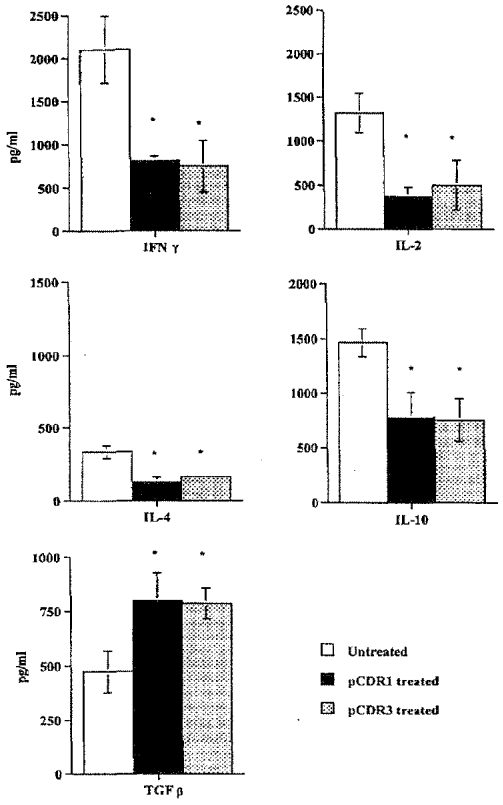

Fig. 4. The effect of treatment with the CDR-based peptides on the cytokine profile. Five-month-old (NZB × NZW)F_1 mice were treated with either pCDR1 or pCDR3, 250 μg/mouse given s.c. in PBS, once a week for 10 weeks. Mice were sacrificed after 5 weeks and at the end of treatment, and the cytokines secreted by their Con A-stimulated splenocytes were measured by ELISA as described in Methods. Summary of results of two experiments, obtained with supernatants of mice sacrificed after 5 weeks of treatment. *$P < 0.05$ as compared to untreated groups.

Treatment of (NZB × NZW)F_1 mice with the CDR-based peptides affects their cytokine profile

Imbalance in the cytokine network plays an important role in the induction and development of experimental SLE (12–14). It was therefore of interest to find out whether treatment with the CDR-based peptides affects the cytokine production by splenocytes of the treated mice. Thus, 5-month-old (NZB × NZW)F_1 mice (10–12 mice per group) were treated weekly for 10 weeks with 250 μg/mouse of either pCDR1 or pCDR3 given s.c. in PBS.

Groups of mice were sacrificed after 5 weeks of treatment and at the end of treatment, and the cytokines secreted by their Con A-stimulated splenocytes were measured. Figure 4 demonstrates the cytokine secretion by splenocytes of untreated mice, and of pCDR1- and pCDR3-treated mice as measured following 5 weeks of treatment. It can be seen in Fig. 4 that the treatment reduced the secreted IL-2 and IFN-γ (T_h1-type) as compared to the untreated group. A lower production of IL-4 and IL-10 (reported to play a role in the pathogenesis of SLE) was also observed in the groups of mice treated with the CDR-based peptides. In contrast to the down-regulation of the T_h1- and T_h2-type cytokines, the secretion of the immunosuppressive cytokine, TGF-β, was up-regulated. Similar results were obtained when the pattern of secreted cytokines was measured at the end of treatment with the CDR-based peptides. The immunomodulation of cytokine secretion by the CDR-based peptides appears to be specific, because peptides with the reversed sequence of the CDR-based peptides did not affect significantly the secretion of the tested cytokines. A down-regulation of 5–10% in the secretion of IFN-γ, IL-2, IL-4 and IL-10 could be observed in supernatants of splenocytes taken from mice treated with the relevant reversed peptides. Occasionally treatment with the reversed peptides resulted in an increased production (by up to 20%) of the later cytokines as compared to the levels of cytokines in supernatants of splenocytes of untreated (NZB × NZW)F_1 mice. Further, no significant increase in active TGF-β levels could be determined in supernatants of splenocytes of mice that were treated with the reversed peptides. It is noteworthy that in most cases the levels of the T_h1- and T_h2-type cytokines were immunomodulated by the CDR-based peptides to levels measured in young (2-month-old) mice with no symptoms of

the SLE-like disease. Thus levels of 652, 630, 142 and 1124 pg/ml of IL-2, IFN-γ, IL-4 and IL-10 respectively were measured in supernatants of Con A-activated splenocytes of 2-month-old (NZB × NZW)F_1 mice. These levels are very similar to those determined in supernatants of splenocytes taken from mice that were treated with the CDR-based peptides (Fig. 4). It should be noted that treatment with the CDR-based peptides up-regulated the secretion of TGF-β to levels higher than those secreted by splenocytes of young mice. Intracellular staining of splenocytes of untreated and CDR-based peptide-treated mice revealed also a reduction in the number of cells expressing the various cytokines. Hence, a reduction of 40% in intracellular IL-2 was observed in splenocytes of both pCDR1- and pCDR3-treated mice. A 17 and 11% reduction in intracellular IFN-γ was observed for pCDR1- and pCDR3-treated mice respectively, and a reduction of 40 and 30% for intracellular IL-4 and IL-10 respectively could be demonstrated for splenocytes of mice treated with either CDR-based peptide as compared to cells of untreated mice. Thus, treatment of (NZB × NZW)F_1 mice with the CDR-based peptides down-regulates the secretion of the T_h1- and T_h2-type cytokines, and up-regulates the secretion of the immunosuppressive cytokine, TGF-β. These results were reproducible in two independent experiments.

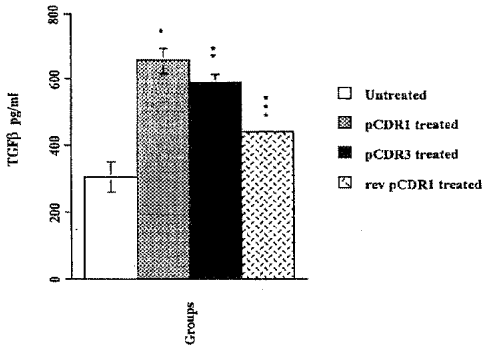

Fig. 5. Levels of TGF-β secreted by spleen cells of young (NZB × NZW)F_1 mice treated with the CDR-based peptides, before being adoptively transferred into 8-month-old diseased mice. Two-month-old (NZB × NZW)F_1 mice were treated 3 times (every other day) with pCDR1, pCDR3 or reversed pCDR1, 300 μg/mouse given s.c. in PBS. Mice were then sacrificed and the levels of active TGF-β secreted by their Con A-stimulated splenocytes were measured by ELISA. Summary of results of two experiments. $^*P = 0.0243$, $^{**}P = 0.0319$, $^{***}P = 0.0987$ as compared with the non-treated group.

Amelioration of the clinical manifestations of (NZB × NZW)F_1 mice by splenocytes of young mice that were treated with the CDR-based peptides

It was of interest to find out whether the beneficial effects of the treatment with the CDR-based peptides could be adoptively transferred with splenocytes of peptide-treated mice. To this end we treated 10-week-old (NZB × NZW)F_1 mice, s.c. 3 times every other day, with pCDR1, pCDR3 or reversed pCDR1, 300 μg/mouse. At the end of this treatment the animals were sacrificed, and their splenocytes were either activated with Con A and tested for the secretion of the immunosuppressive cytokine TGF-β or injected i.p. (20×10^6/ mouse) to respective groups of 8-month-old (NZB × NZW)F_1 mice. Figure 5 shows that the secretion of TGF-β is significantly higher in the splenocytes of pCDR1 ($P = 0.0243$)- or pCDR3 ($P = 0.0319$)-treated groups when compared with the control of untreated mice. TGF-β production by spleen cells from mice treated with reversed pCDR1 was not significantly different from that of control mice ($P = 0.0987$). Table 5 represents the clinical manifestations of the SLE-like disease in the old (NZB × NZW)F_1 mice that were transferred with splenocytes from animals treated with the pCDR peptides. As shown in Table 5 the transfer of splenocytes from pCDR1- and pCDR3-treated mice ameliorates significantly the kidney disease as manifested by proteinuria and the deposits of the immune complexes in the kidneys. This adoptive transfer of splenocytes from peptide-treated mice resulted in a moderate reduction in anti-dsDNA antibody titers (data not shown). Because the recipient mice were sacrificed ~3 weeks following cell transfer, the effect of the inoculated cells on survival could not be determined in these experiments. Nevertheless, whereas in each experiment at least one mouse of the untreated group died before the end of the experiment, all the recipients of splenocytes of mice treated with the CDR-based peptides survived. Thus, beneficial effects of treatment with the CDR-based peptides can be transferred by splenocytes of peptide-treated mice.

Discussion

The main findings of the present report are that treatment of the lupus-like disease of (NZB × NZW)F_1 mice, when clinical

Table 5. Down-regulation of SLE clinical manifestation in (NZB × NZW)F_1 mice by splenocytes of mice treated with the CDR-based peptides

Group	Treatment	Proteinuria (g/l ±SEM)	Immune complex deposits (± SEM)
1	20×10^6 spleen cells from pCDR1-treated mice	0.62 ± 0.2^a	0.80 ± 0.2^c
2	20×10^6 spleen cells from pCDR3-treated mice	1.18 ± 0.2^b	0.90 ± 0.3^d
3	20×10^6 spleen cells from reversed pCDR1-treated mice	8.45 ± 3.4	2.00 ± 0.2

Spleen cells (20×10^6) from (NZB × NZW)F_1 mice injected s.c. 3 times (every other day) with 300 μg/mouse of pCDR1, pCDR3 and reversed pCDR1 were transferred to groups of 10 (NZB × NZW)F_1 mice at the age of 8 months. Two weeks later proteinuria was measured and the immune complex deposits were assessed at sacrifice.

$^{a,b}P \leq 0.04$; $^{c,d}P \leq 0.02$ as compared with the group treated with reversed pCDR-1.

symptoms were already developed, ameliorated disease manifestations in all measured parameters. Moreover, some beneficial effects could be adoptively transferred into (NZB × NZW)F_1 mice with the full-blown SLE-like disease by splenocytes of young (NZB × NZW)F_1 mice that were treated with the CDR-based peptides. The observed benefits were associated with a down-regulation of IL-2, IFN-γ, IL-4 and IL-10, and an up-regulation in the production of the immunosuppressive cytokine, TGF-β. The elevated production of TGF-β was determined in splenocytes of mice treated with the CDR-based peptides that could transfer ameliorating effects of the peptides.

Anti-DNA antibodies play a central role in the diagnosis of SLE. Indeed, the most characteristic serological abnormality found in patients with SLE is the presence of anti-dsDNA antibodies (15,16). Anti-DNA antibodies are considered instrumental in the pathogenesis of the immune glomerulonephritis in SLE in mice and in human patients (15). In (NZB × NZW)F_1 mice as well as in other SLE models, the pathogenic anti-DNA antibodies were reported to be of the IgG2a and IgG3 isotypes (2,17,18). The latter compose the immune complexes that lead to glomerulonephritis. This kidney disease is the major cause of death in SLE afflicted mice (2). Indeed, our results demonstrate that mice treated with the pCDR1 or pCDR3 had lower titers of anti-DNA antibodies of these pathogenic isotypes in their sera and less immune complex deposits of the latter isotypes in their kidneys, and a better survival rate.

Most of the experimental protocols of the present report were not designed to compare mortality rates between the groups that were treated with the CDR-based peptides and the non-treated mice. Therefore, mice were sacrificed in most experiments at the age of 8 months, before death occurred. Nevertheless, when mice were treated for 10 weeks starting at the age of 7 months, it could be seen that mortality was significantly lower in the peptide-treated groups (Table 4). The higher survival rate could be attributed to the amelioration in the renal disease that has a central role in disease course in animal and human SLE.

The use of peptides for treating the lupus-like disease of (NZB × NZW)F_1 mice has been recently reported by other laboratories as well. Most of the studies report the successful prevention of SLE manifestations as we have previously reported (11) and not treatment of an already existing disease. Thus, injection of a peptide based on the CDR3 of an anti-DNA antibody from naive BALB/c mice to (NZB × NZW)F_1 mice, at the age of 2 months, resulted in a delay in mortality rate and onset of proteinuria (19). Further, induction of tolerance in young (NZB × NZW)F_1 mice by i.v. injections of peptides derived from the heavy chain variable (V_H) regions of anti-DNA autoantibodies postponed the development of the SLE-like disease in these mice (20). Peptides based on a nephritogenic (R4A) anti-DNA antibody were shown to protect mice from renal deposition of anti-DNA antibodies (21). In agreement with our present study demonstrating the beneficial effects of treatment with the CDR-based peptides on an already established SLE, Kaliyaperumal *et al.* (22) reported the effectiveness of treatment with nucleosomal histone peptides not only in delaying the onset of disease, but also in halting the progression of an established renal disease in (SWR × NZB)F_1 lupus-prone mice. Further, treatment with a peptide ['consensus peptide' (pCONS)] based on an algorithm that defines the T cell stimulatory amino acid sequences from the V_H regions of multiple (NZB × NZW)F_1 IgG antibodies to DNA was effective when given to diseased mice (23). Hence, the reported, as well as our, findings support the crucial effect of heavy chain variable region derived peptides in the modulation of murine lupus.

Cytokines have been suggested to play an important role in the immune dysregulation observed in lupus-prone mice and in SLE patients (14,24). Treatment with the CDR-based peptides reduced the levels of IL-2 and IFN-γ. While the role of IL-2 in SLE has not been conclusively determined (14), the role of IFN-γ in the pathogenesis of lupus has been consistently reported. Thus, treatment of (NZB × NZW)F_1 mice with IFN-γ accelerated disease, whereas treatment with anti-IFN-γ antibody (25) or soluble IFN-γ receptor (26) delayed disease progression. A similar role for IFN-γ was reported for MRL-*lpr/lpr* mice in which deficiency of the IFN-γ gene (27) or the IFN-γ receptor gene (28) protected from disease development. We demonstrated that treatment with the CDR-based peptides down-regulated IL-10 and IL-4 secretion. Increased IL-10 production has been reported in all SLE-prone mice (14) and treatment with IL-10 accelerated disease manifestations, whereas administration of anti-IL-10 delayed onset of SLE (29). As for IL-4, it has been reported that transfer of IL-4-stimulated splenocytes into syngeneic recipients increased anti-DNA production, and administration of anti-IL-4 inhibited the autoantibody production and prevented glomerulonephritis (14). Hahn *et al.* (23) demonstrated significantly lower levels of IFN-γ and IL-4 in plasma of (NZB × NZW)F_1 treated with pCONS as compared to plasma of saline-treated mice, supporting the results of the present report. In contrast to the reduced production of the above cytokines following treatment with the CDR-based peptides, treatment with either pCDR1 or pCDR3 resulted in the secretion of elevated levels of the immunosuppressive cytokine, TGF-β. In agreement, it has been reported that injection of a TGF-β cDNA expression vector into the skeletal muscle of the lupus-prone MRL-*lpr/lpr* mice decreased autoantibody production (30). Furthermore, infection of (NZB × NZW)F_1 mice with *Plasmodium chabaudi* led to an improvement in the clinical lupus-like symptoms of the mice. The latter was associated with an increased mRNA expression of TGF-β (31). Both constitutive and stimulated levels of TGF-β are lower in patients with SLE, and the high IgG production is attributed, in part, to low levels of TGF-β (32). The ability of splenocytes of young (NZB × NZW)F_1 mice that were treated with the CDR-based peptides to adoptively transfer their capacity to down-regulate SLE manifestations (Table 5) suggests that one of the mechanisms by which the peptides exert their ameliorating effects is the induction of an immunosuppresive response mediated by regulatory cells (33) and/or by immunosuppresive cytokines like TGF-β. As a result of that, the secretion of cytokines supporting SLE-associated autoimmune responses is actively suppressed. Indeed, splenocytes of mice that were treated with the CDR-based peptides were shown to secrete elevated levels of TGF-β (Fig. 5).

Taking together, the reported results indicate that a relatively short course of treatment with the CDR-based peptides ameliorates the clinical manifestations of an estab-

lished SLE in (NZB × NZW)F_1 mice. The treatment with the peptides was shown to be effective in SLE-prone mice [(11) and this report] as well as in a model of induced experimental SLE (9). Thus, the peptides pCDR1 and pCDR3 might be potential candidates for the treatment of human SLE.

Acknowledgements

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Abbreviations

CDR	complementarity-determining region
Con A	concanavalin A
pCDR	peptide based on CDR
SLE	systemic lupus erythematosus
TGF	transforming growth factor

References

1 Hahn, B. H. 1993. An overview of the pathogenesis of systemic lupus erythematosus. In Wallace, D. J. and Hahn, B. H., eds, *Dubois' Lupus Erythematosus*, p. 69. Williams & Wilkins, Philadelphia, PA.
2 Theofilopoulos, A. N. 1992. Murine models of lupus. In Lahita, R. G., ed., *Systemic Lupus Erythematosus*, p. 121. Churchill Livingston, New York.
3 Mendlovic, S., Brocke, S., Shoenfeld, Y., Ben Bassat, M., Meshorer, A., Bakimer, R. and Mozes, E. 1988. Induction of a systemic lupus erythematosus-like disease in mice by a common human anti-DNA idiotype. *Proc. Natl Acad. Sci. USA* 85:2260.
4 Waisman, A., Mendlovic, S., Ruiz, J. P., Zinger, H., Meshorer, A. and Mozes, E. 1993. The role of the 16/6 idiotype network in the induction and manifestation of systemic lupus erythematosus. *Int. Immunol.* 5:1293
5 Tillman, D. M., Jou, N. T., Hill, R. J. and Marion, T. N. 1992. Both IgM and IgG anti-DNA antibodies are the products of clonally selective B cell stimulation in (NZB × NZW)F_1 mice. *J. Exp. Med.* 176:361.
6 Waisman, A. and Mozes, E. Variable region sequences of autoantibodies from mice with experimental systemic lupus erythematosus. *Eur. J. Immunol.* 23:1566.
7 Wloch, M. K., Alexander, A. L., Pippen, A. M. N., Pisetsky, D. S. and Gilkson, D. S. 1997. Molecular properties of anti-DNA induced in preautoimmune NZB/W mice by immunization with bacterial DNA. *J. Immunol.* 158:4500.
8 Waisman, A., Ruiz, P. J., Israeli, E., Eilat, E., Könen-Waisman, S., Zinger, H., Dayan, M. and Mozes, E. 1997. Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of a pathogenic anti-DNA monoclonal antibody. *Proc. Natl Acad. Sci. USA* 94:4620.
9 Eilat, E., Dayan, M., Zinger, H. and Mozes, E. 2001 The mechanism by which a peptide based on the complementarity determining region-1 of a pathogenic anti- DNA autoantibody ameliorates experimental SLE. *Proc. Natl Acad. Sci. USA* 98:1148.
10 Eilat, E., Zinger, H., Nyska, A. and Mozes, E. 2000. Prevention of systemic lupus erythematosus-like disease in (NZB × NZW)F_1 mice by treating with CDR1-and CDR3-based peptides of a pathogenic autoantibody. *J. Clin. Immunol.* 20:268.
11 Schnolzer, M., Alewood, P. F. and Kent, S. B. H. 1992. *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Pept. Protein Res.* 40:180.
12 Handwerger, B. S., Rus, V., da Silva, L. and Via, C. S. 1994. The role of cytokines in the immunopathogenesis of lupus. *Semin. Immunopathol.* 16:153.
13 Segal, R., Bermas, B. L., Dayan, M., Kalush, F., Shearer, G. M. and Mozes, E. 1997. Kinetics of cytokine production in experimental systemic lupus erythematosus: involvement of T helper cell 1/T helper cell 2-type cytokines in disease. *J. Immunol.* 158:3009.
14 Theofilopoulos, A. N., and Lawson, B. R. 1999. Tumour necrosis factor and other cytokines in murine lupus. *Ann. Rheum. Dis.* 58:149.
15 Hahn, B. H. 1998. Antibodies to DNA. *N. Engl. J. Med.* 338:1359.
16 Spronk, P. E., Horst, G., VabDerGun, B. T., Limburg, P. C. and Kallenberg, C. G. 1996. Anti-dsDNA production coincides with concurrent B and T cell activation during development of active disease in systemic lupus erythematosus (SLE). *Clin. Exp. Immunol.* 104:446.
17 Izui, S., Iwamoto, M., Fossati, L., Merino, R., Takahashi, S. and Ibanou-Zekri, N. 1995. The Yaa gene model of systemic lupus erythematosus. *Immunol. Rev.* 144:137.
18 Dayan, M., Zinger, H., Kalush, F., Mor, G., Amir Zaltzman, Y., Kohen, F., Sthoeger, Z. and Mozes, E. 1997. The beneficial effects of treatment with tamoxifen and anti-oestradiol antibody on experimental systemic lupus erythematosus are associated with cytokine modulations. *Immunology* 90:101.
19 Jouanne, C., Avrameas, S. and Payelle-Brogard, B. 1999. A peptide derived from a polyreactive monoclonal anti-DNA natural antibody can modulate lupus development in (NZB × NZW)F_1 mice. *Immunology* 96:333.
20 Singh, R. R., Ebling, F. M., Sercarz, E. E. and Hahn, B. H. 1995. Immune tolerance to autoantibody-derived peptides delays development of autoimmunity in murine lupus. *J. Clin. Invest.* 96:2990.
21 Gaynor, B., Putterman, C., Valadon, P., Spatz, L., Scharff, M. and Diamond, B. 1997. Peptide inhibition of glomerular deposition of an anti-DNA antibody. *Proc. Natl Acad. Sci. USA* 94:1955.
22 Kaliyaperumal, A., Michaels, M. A. and Datta, S. K. 1999. Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: tolerance spreading impairs pathogenic function of autoimmune T and B cells. *J. Immunol.* 162:5775.
23 Hahn, B. H., Singh, R. S., Wong, W.-K., Tsao, B. P., Bulpitt,K. and Ebling,F. M. 2001. Treatment with a consensus peptide based on amino acid sequences in autoantibodies prevents T cell activation by autoantigens and delays onset in murine lupus. *Arthritis Rheum.* 44:432.
24 Dean, G. S., Tirrell-Price, J., Crawley, E. and Isenberg, D. A. 2000. Cytokines and systemic lupus erythematosus. *Ann. Rheum. Dis.* 59:243.
25 Jacob, C. O., van der Meide, P. H. and McDevitt, H. O. 1987. *In vivo* treatment of (NZB × NZW)F_1 lupus-like nephrithis with monoclonal antibody to gamma interferon. *J. Exp. Med.* 166:798.
26 Ozmen, L., Roman, D., Fountoulakis, M., Schmid, G., Ryffel, B. and Garotta, G. 1995. Experimental therapy of systemic lupus erythematosus: the treatment of NZB/W mice with mouse soluble interferon-gamma receptor inhibits the onset of glomerulonephritis. *Eur. J. Immunol.* 25:6.
27 Balomenos, D., Rumold, R. and Theofilopoulos, A. N. 1998. Interferon-γ is required for lupus-like disease and lymphoaccumulation in MRL-*lpr* mice. *J. Clin. Invest.* 101:364.
28 Peng, S. L., Moslehi, J. and Craft, J. 1997. Roles of interferon-γ and interleukin-4 in murine lupus. *J. Clin. Invest.* 99:1936.
29 Ishida, H., Muchamuel, T., Sakaguchi, S., Andrade, S., Menon, S. and Howard, M. 1994. Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F_1 mice. *J. Exp. Med.* 179:305.
30 Raz, E., Watanabe, A., Baird, S. M., Isenberg, R. A., Parr, T. B., Lotz, M., Kipps, T. J. and Carson, D. A. 1993. Systemic immunological effects of cytokine genes injected into skeletal muscle. *Proc. Natl Acad. Sci. USA* 90:4523.
31 Sato, M. N., Minoprio, P., Avrameas, S. and Terninck, T. 2000. Changes in the cytokine profile of lupus-prone mice (NZB/NZW)F_1 induced by *Plasmodium chaubaudi* and their implications in the reversal of clinical symptoms. *Clin. Exp. Immunol.* 119:333.
32 Ohtsuka, K., Dixon Gray, J., Stimmler, M. M., Toro, B. and Horwitz, D. A. 1998. Decreased production of TGF-β by lymphocytes from patients with systemic lupus erythematosus. *J. Immunol.* 160:2539.
33 Shevach, EM. 2001. Certified professionals: $CD4^+CD25^+$ suppressor T cells. *J. Exp. Med.* 193:F41.